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(54) Title: INHIBITION OF CELL GROWTH BY KERATAN SULFATE, CHONDROITIN SULFATE, DERMATAN SULFATE AND OTHER GLYCANS

(57) Abstract

The present invention is directed to methods of using keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, and hyaluronic acid, and molecules and compositions comprising keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, and hyaluronic acid, to inhibit or prevent neurite outgrowth, i.e., axonal growth, or nerve regeneration (colectively termed herein "nerve growth"), or glial cell migration or invasion, or regeneration, and therapeutically, where the foregoing is desired. In another embodiment of the invention, inhibitors and antagonists of keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, and hyaluronic acid, and molecules and compositions containing the same, may be used to promote nerve growth or glial cell migration or invasion, and can be administered therapeutically. Such inhibitors and antagonists include but are not limited to antibodies, degradative enzymes, lectins; and disaccharide antagonists of the receptors for keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin or hyaluronate.

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INHIBITION OF CELL GROWTH BY KERATAN SULFATE, CHONDROITIN SULFATE, DERMATAN SULFATE AND OTHER GLYCANS

1. INTRODUCTION

The present invention relates to compositions comprising keratan sulfate, chondroitin sulfate, or dermatan sulfate, also heparan sulfate, heparin, or hyaluronic acid (hyaluronate), or any combination of these molecules--in particular, glycosaminoglycans or proteoglycans--and the uses of such compositions in inhibition of neurite outgrowth and glial cell invasion or migration. The invention also relates to compositions comprising an antagonist of inhibition mediated by keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, or hyaluronate such as antibodies to keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin or hyaluronate; enzymes that degrade keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, or hyaluronic acid; lectins specific for keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, or hyaluronic acid, or disaccharide antagonists of the receptors for keratan sulfate, chondioitin sulfate, dermatan sulfate, heparan sulfate, heparin or hyaluronic acid; and the uses of such compositions for promotion of cell growth or regeneration, in particular, neurite outgrowth or glial cell migration. Therapeutic uses of the compositions of the invention are provided.

2. BACKGROUND OF THE INVENTION

2.1. AXONAL GROWTH

Axons grow in stereotyped patterns toward their targets during development of the nervous system. During this directed elongation, axonal growth cones undergo multiple interactions with components of the environment such as the extracellular matrix (Carbonetto et al., 1982, Science 216:897-899; Hankin, M. H. and Silver, J., 1986, Mechanisms of axonal guidance: the problem of intersecting fiber systems, in The Cellular Basis of Morphogenesis, (Leon W. Browder, Ed.) Vol. 2:565-599, Plenum Publishing Corp., New York, New York; Mirsky et al., 1986, J. Neurocytology 15(6):799-815; Rogers et al., 1986, Devel. Biol. 113:429-435; Antonicek et al., 1987, J. Cell Biol. 104(6):1587-1595; Bork et al., 1987, J. Comp. Neurol. 264:147-158; Liesi, P. and Silver, J., 1988, Devel. Biol. 130:774-785; Lumsden, A. and Keynes, R., 1989, Nature 337:424-428), the surfaces of neuroepithelial cell endfeet (Silver, J., and Sapiro, J., 1981, J. Comp. Neurol. 202:521-538; Silver, J., and Rutishauser, U., 1984, Devel. Biol. 106:485-499; Bastiani, M. J. and Goodman, C.S., 1986, J. Neurosci. 6(12):3542-3551; Kuwada, J. Y., 1986, Science 233:740-746; Holley, J. A., 1987, Devel. Biol. 123:389-400; Holley, J. and Silver, J., 1987, Devel. Biol. 123:375-388), other axons (Rutishauser et al., 1978, J. Cell Biol. 79:382-393; Fushiki, S., and Schachner, M., 1986, Brain Res. 389:153-167) and glia (Silver, J., and Robb, R. M., 1979, Devel. Biol. 68:175-190; Singer, et al., 1979, J. Comp. Neurol. 185:1-22; Simpson, S., 1983, in Spinal Cord Reconstruction, Raven Press, New York, New York pp. 151-162; Silver, J., 1984, J. Comp. Neurol. 223:238-251; Poston et al., 1985, Society for Neuroscience Abstract, 11:584; Bastiani, M. J. and Goodman, C.S., 1986, supra; Cooper, N. G. F., and Steindler, D. A., 1986, Brain Res. 380:341-348; Poston et al., 1987, The Making of the Nervous System,

London: Longmans). It has been suggested that the adhesive properties of these various surfaces lure axons along their appropriate pathways (Letournau, P. C., 1975, Devel. Biol. 44:92-101; Sidman, R. L., and Wessells, N. K., 1975, Exp. Neurol. 48:237-251; Constantine-Paton, M., 1983, Devel. Biol. 97:239-244; Rogers et al., 1983, Devel. Biol. 98:212-220; Gundersen, R. W., and Park, K. H. C., 1984, Devel. Biol. 104:18-27; Silver, J., and Rutishauser, U., 1984, Dev. Biol. 106:485-499; Tomaselli, K. J. et al., 1986, J. Cell Biol. 103:2659-2672). Local cues organized into gradients may be important in providing directionality as well (Lumsden, A. G. S., and Davies, A., 1983, Nature (London) 306:786-788). In fact, many or all of these factors could be interacting, in concert, to promote axon growth along a proper trajectory at various stages of development.

Recent findings indicate that mechanisms exist which inhibit or repel axons and they may be as important for axon guidance as are adhesive or attractive mechanisms (Silver, J., and Sapiro, J., 1981, J. Comp. Neurol. 202:521-538; Haydon et al., 1984, Science 226:561-564; Poston et al., 1985, Society for Neurosciences Abstract 11:584; Kapfhammer, J. P., and Raper, J. A., 1987 J. Neurosci. 7(5):1595-1600; Kapfhammer, J. P., and Raper, J. A., 1987, J. Neurosci. 7(1):201-212; Perris, R., and Johansson, S., 1987, J. Cell Biol. 105(6):2511-2521; Silver et al., 1987, J. Neurosci. 7(7):2264-2272; McCobb et al., 1988, J. Neurosci. Research 19:19-26; Patterson, P. H., 1988, Neuron 1:263-267; Schwab, M. E., and Caroni, P., 1988, J. Neurosci. 8(7):2381-2393; Tosney, K., 1988, Devel. Biol. 127:266-286; Webster et al., 1988, J. Comp. Neurol. 269:592-611; Gurwitz, D. and Cunningham, D. D., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:3440-3444). Inhibitory components may take the form of cellular boundaries or barriers along an axon pathway (Silver, J., 1984, J. Comp.

Neurol. 223:238-251) and they may act by mechanical as well as chemical means (Silver, J., and Rutishauser, U., 1984, supra; Tosney, K. and Landmesser, L., 1985, Devel. Biol. 109:193-214; Silver et al., 1987, J. Neurosci. 7:2264-2272; Stern, C. D., and Keynes, R. J., 1987, Development, 99:261-272; Webster, M. J. et al., 1988, J. Comp. Neurol. 269:592-611). Axon inhibition can occur between different classes of neurons (Kapfhammer, J. P., and Raper, J. A., 1987, J. Neurosci. 7(1):201-212), in association with glial cells (Silver et al., 1982, J. Comp. Neurol. 210:10-29; Silver, J., 1984, J. Comp. Neurol. 223:228-251; Poston et al., 1985, Society for Neuroscience Abstract 11:584; Silver et al., 1987, supra; Steindler, D. A., and Cooper, N. G. F., 1987, Devel. Brain Res. 36:27-38; Schwab, M. E., and Caroni, P., 1988, J. Neurosci. 8(7):2381-2393), in response to mesenchymal components (Keynes, R. J., and Stern, C. D., 1984, Nature (London) 310:786-789; Tosney, K., 1988, Devel. Biol. 127:266-286) or to soluble factors (Haydon et al., 1984, Science 226:561-564; Verna, J. M., 1985, J. Embryol. Exp. Morphol. 86:53-70; McCobb et al., 1988, J. Neurosci. Res. 19:19-26). As yet, we have only a limited understanding of axon barriers. One would like to know what they are made of, how they function, and what happens at the cellular and molecular levels when axons encounter them.

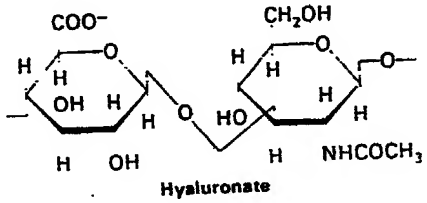
One region of the central nervous system that may be a barrier to axon growth is the roof plate, located at the dorsal midline of the developing vertebrate spinal cord (His, W., 1891, I. Verlangertes Mark, 29:1-74; Ramon y Cajal, S., 1911, Histologie du systeme nerveux de l'homme et des vertebres. (francaise rev. et mise a jour par l'auteur, Ed.) Vol. 1. A., Maloine, Paris). This region is comprised of primitive glial cells as determined morphologically (His, 1891, supra), by the use of tritiated thymidine autoradiography (Altman, J., and Bayer, S. A.,

1984, in Advances in Anatomy, Embryology and Cell Biology, Vol. 85, pp. 53-83, Springer-Verlag, Heidelberg, Germany) and with the use of antibodies RC1 and RC2 which specifically label embryonic radial glial cells (Edwards et al., 1986, 8C7, Society for Neuroscience Abstract 12:182). The roof plate contains transient channels which are first observed as a single row of extracellular spaces at E9 (Snow, D., et al., 1987, Society for Neurosciences Abstract 13(1):1987). The roof plate undergoes a gradual change in morphology between E12.5, when it has a wedge shape, and E15.5 when it becomes a long, thin septum-like structure at the dorsal midline of the spinal cord in rat. A dorsal subpopulation of the early ventral commissural axons as well as primary afferents from the dorsal root ganglia come in close proximity to the roof plate. Even though both axon systems have potential targets or pathways in the contralateral spinal cord, they do not cross the roof plate to reach them. Figure 1 is a schematic diagram which depicts the relationship of the commissural and sensory axon systems to the roof plate at E13.5 and E15.5 in rat. However, at a later stage of embryonic development in rat (Smith, C. L., 1983, J. Comp. Neurol. 220:29-43) and in frog (Nordlander, R. and Singer, M., 1982, Exp. Neurol. 75:221-228), a population of sensory axons do cross the dorsal spinal cord just below the posterior columns to form the dorsal commissure.

2.2. PROTEOGLYCANS

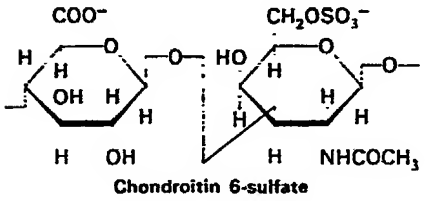
Proteoglycans are molecules found in abundance in connective tissue, which consist of about 50-95% polysaccharide and about 5-50% protein. Glycosaminoglycans are the polysaccharide chains of proteoglycans, and contain repeating units of disaccharides which consist of an aminosugar derivative, either glucosamine or galactosamine.

A negatively charged carboxylate or sulfate group is found in at least one of the sugar units of the disaccharide. Common glycosaminoglycans include hyaluronate (HA), chondroitin sulfate (CS), keratan sulfate (KS), dermatan sulfate (DS), heparan sulfate (HS), and heparin (HN). The structural formulas for the disaccharide unit of hyaluronic acid, chondroitin 6-sulfate, keratan sulfate, dermatan sulfate, and heparin are as follows (note that forms exist with various degrees of sulfation greater than those shown):



Hyaluronate

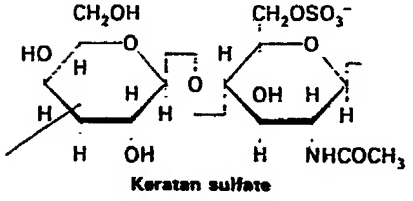
hyaluronic acid



Chondroitin 6-sulfate

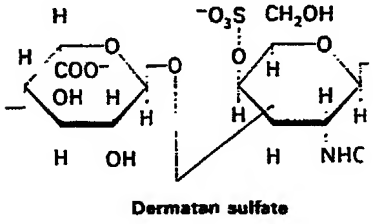
chondroitin 6-sulfate

keratan sulfate

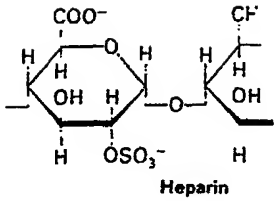


Keratan sulfate

dermatan sulfate



Dermatan sulfate



Heparin

heparin

The glycosaminoglycan chains of proteoglycans are found covalently attached to a polypeptide backbone called the core protein (Stryer, L., 1981, Biochemistry, 2d ed., W. H. Freeman & Co., New York, pp. 200-203).

Combinations of proteoglycans and growth-promoting molecules exist in many regions where pioneering axons elongate. Laminin as well as neural cell adhesion molecule (NCAM) are present in tandem with proteoglycan along the developing optic pathway (Liesi, P., and Silver, J., 1988, Dev. Biol. 130:774-785; Silver and Rutishauser, 1984, Dev. Biol. 106:485-499; Bork et al., 1987, J. Comp. Neurol. 264:147-158).

In other localization studies, Tosney and Landmesser (1985, Dev. Biol. 109:193-214) showed that the posterior sclerotome contains high levels of glycosaminoglycans, as determined by Alcian Blue staining, and that growth cones do not explore these regions. Funderburg et al. (1986, Dev. Biol. 116:267-277) have confirmed the presence of keratan sulfate in the outer epidermis. Studies in chick forebrain have shown that chondroitin-6-sulfate is present in large quantities in channels beneath the cortical plate region where axons are not found (Palmert et al., 1986, Society for Neurosciences Abstract 12(2):1334). A keratan sulfate proteoglycan has been identified in the rat cerebral cortex (Vitello et al., 1978, Biochim. Biophys. Acta 539:305-314) and in corpora amylacea of human brain (Liu, H. M. et al., 1987, J. Neuroimmunol. 14:49-60).

2.3. PROTEOGLYCAN REGULATION OF CELL GROWTH

Different proteoglycans have been shown to exert a wide spectrum of effects on the migratory behavior of a variety of different cell types (Walicke, P.A., 1988, Exp. Neurol. 102:144-148; Daman et al., 1988, J. Cell Physiol. 135:293-300). Perris and Johansson (1987, J. Cell Biol. 105:2511-2521) have shown that a form of chondroitin sulfate proteoglycan is inhibitory to the migration of neural crest cells in vitro.

Specific glycosaminoglycans/proteoglycans have been shown to have inhibitory effects on neurite outgrowth and cell attachment in vitro. Carbonetto et al. (1983, J. Neurosci. 3(11):2324-2335) showed that chondroitin sulfate, hyaluronic acid, and heparin inhibit chick dorsal root ganglion and PC-12 axon outgrowth on fibronectin in a three-dimensional HEMA-gel culture system. Support of neurite outgrowth by fibronectin was significantly reduced by the addition of heparin to a HEMA/fibronectin gel. This proteoglycan, in high concentrations, also inhibited the attachment and neurite formation of human neuroblastoma cells on a cholera toxin B/ganglioside GM1-binding substratum (Mugnai et al., 1988, Exp. Cell Res. 175:299-247).

Unfractionated cartilage proteoglycans, though to a lesser extent a purified cartilage component, chondroitin sulfate, were found to inhibit fibroblast binding to collagen and fibronectin in vitro (Rich, et al., 1981, Nature 293:224-226). Dermatan sulfate proteoglycan (DS-PG) was observed to inhibit the attachment and spreading of 3T3 fibroblasts on plasma fibronectin-coated culture substrata (Lewandowska et al., 1987, J. Cell Biol. 105:1443-1454). Previously, however, glycosaminoglycans (GAGs), principally heparan sulfate and dermatan sulfate, were identified as mediators of fibroblast (murine 3T3 cell) attachment to fibronectin. The heparan and dermatan

GAGs bound to serum fibronectin covalently attached to Sepharose, while other proteoglycans, notably various chondroitin sulfates and under-sulfated heparan sulfate, did not bind to the column (Laterra, et al., 1980, Proc. Natl. Acad. Sci. U.S.A. 77:6662-6666).

Sulfonated glycosaminoglycans have been reported to reduce the ability of bacteria to adhere to urinary bladder mucosa, as part of an anti-bacterial defense mechanism (Parsons, C. L., 1986, Urologic Clinics of North America 13(4):563-568; Parsons, C. L., et al., 1981, J. Infec. Dis. 144(2):180; Parsons, C. L., et al., Science 208:605-607; Hanno, P. M., et al., 1978, J. Surg. Res. 25:324-329; Parsons, C. L., et al., 1978, Am. J. Pathol. 93(2):423-432).

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery that keratan sulfate (KS), chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin (HN) and/or hyaluronic acid (HA) can inhibit neurite outgrowth, i.e., axonal growth, and glial cell migration or invasion. Neurite outgrowth, i.e., axonal growth, and nerve regeneration herein may be referred to as "nerve growth."

Presence of these glycans inhibits neurite outgrowth even in the presence of nerve growth-promoting factors such as laminin and NCAM. These glycans prevent glial cell, in particular astrocyte, migration or invasion on laminin. Accordingly, the present invention is directed to methods of using keratan sulfate, and molecules and compositions comprising keratan sulfate, to inhibit or prevent neurite outgrowth and/or glial cell migration or invasion, or nerve or glial cell regeneration. The methods to inhibit neurite outgrowth or glial cell migration or invasion may be used therapeutically, where that is desired. Such molecules comprising keratan sulfate include

but are not limited to keratan sulfate glycosaminoglycan and keratan sulfate proteoglycan, with keratan sulfate proteoglycan most preferred. The invention is further directed to molecules and compositions comprising chondroitin sulfate, and the therapeutic uses thereof to inhibit or prevent neurite outgrowth or glial cell migration or invasion. Molecules comprising chondroitin sulfate include but are not limited to chondroitin sulfate glycosaminoglycan, or more preferably, chondroitin sulfate proteoglycan. The invention also encompasses molecules and compositions comprising dermatan sulfate and the therapeutic uses thereof to inhibit or prevent neurite outgrowth or glial cell migration or invasion. Molecules of dermatan sulfate include but are not limited to dermatan sulfate glycosaminoglycan, or more preferably dermatan sulfate proteoglycan.

In another embodiment, inhibitors and antagonists of keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and/or hyaluronic acid, and molecules and compositions containing the same, may be used to promote neurite outgrowth or glial cell migration or invasion and can be administered therapeutically. Such inhibitors and antagonists include but are not limited to antibodies to KS, CS, DS, HS, HN or HA, and derivatives or fragments thereof, enzymes that degrade KS, CS, DS, HS, HN or HA, lectins specific for KS, CS, DS, HS, HN or HA, and disaccharide antagonists of receptors specific for KS, CS, DS, HS, HN or HA. In this embodiment, promotion of neurite outgrowth and glial cell migration or invasion occurs by removing the inhibitory influence of molecules comprising KS, CS, DS, HS, HN or HA, thus allowing promotion of neurite outgrowth or glial cell migration or invasion by endogenous or exogenously added molecules.

In a further embodiment, molecules comprising keratan sulfate can be used together with molecules comprising another glycosaminoglycan or the disaccharide unit thereof, preferably chondroitin sulfate, in the methods of the invention.

The present invention also provides pharmaceutical compositions comprising effective amounts of the molecules and compositions comprising keratan sulfate and/or chrondroitin sulfate, dermatan sulfate, heparan sulfate, heparin or hyaluronate.

As detailed in the examples sections infra, immunocytochemical localization data is presented which indicates that keratan sulfate, alone or in combination with other molecules such as chondroitin sulfate, may be in part responsible for the inhibition of axon elongation through the roof plate in the embryonic spinal cord. In a further example, we demonstrate in vitro that keratan sulfate/chondroitin sulfate proteoglycan or dermatan sulfate proteoglycan actively inhibits neurite elongation in a concentration dependent manner. The examples also indicate that dermatan sulfate and keratan sulfate/chondroitin sulfate inhibit invasion or migration of glial cells, including astrocytes, in a concentration dependent manner.

3.1. DEFINITIONS

As used herein, the following terms shall have the meanings indicated:

ChE:	cholinesterase
CS:	chondroitin sulfate
DRG:	dorsal root ganglion
DS-PG:	dermatan sulfate proteoglycan
E11.5:	embryonic day 11.5
GAG:	glycosaminoglycan
HA:	hyaluronic acid, hyaluronate

HN:	heparin
HRP:	horseradish peroxidase
HS:	heparan sulfate
Ig:	immunoglobin
KS:	keratan sulfate
KS/CS-PG:	keratan sulfate/chondroitin sulfate proteoglycan
FITC:	fluorescein isothiocyanate
LN:	laminin
NCAM:	neural cell adhesion molecule
NGS:	normal goat serum
P3:	postnatal day 3
PBS:	phosphate buffered saline
PG:	proteoglycan
RCS:	rat chondrosarcoma tumor cell line cartilage chondroitin sulfate proteoglycan
RITC:	rhodamine isothiocyanate
TBS/BSA:	Tris-buffered saline/bovine serum albumin
TPA:	tetragonolobus purpureas agglutinin (lotus tetragonolobus, lotus lectin)

4. DESCRIPTION OF THE FIGURES

Figure 1. Schematic diagram of embryonic day 13.5 (E13.5) and E15.5 rat cervical spinal cord. The relationship of the roof plate (RP) to the developing dorsal column (sensory) axons (SA) and the commissural axons (CA) is depicted. The earliest dorsal population of commissural axons originate near the roof plate. The axons elongate dorsolaterally, then travel ventrally near the periphery of the cord to decussate at the floor plate (FP). The primary sensory afferents (SA) wait in the dorsal root entry zone in an oval bundle on E13.5, travelling rostrally

and caudally for a few segments. The dorsal column axons move medially with development and abut the roof plate by E15.5. Like the commissural axons, the dorsal column axons respect the dorsal midline barrier.

Figure 2. (A) Traverse 1 μm plastic section of E11.5 rat cervical spinal cord. The roof plate (RP) cells are beginning to form the wedge shape which will become most apparent on E13.5. The cells are arranged in an arching pattern in comparison to adjacent neuroepithelial cells which are more linear. Extracellular space between the presumptive roof plate glial cells is not yet significant in comparison to the spaces seen betwen the cells of the remainder of the cord (compare with Fig. 3A); cc, central canal, (250X), (B) A 10 μm cryostat section of rat cervical spinal cord on day E11.5 labelled with 1C12 antibody which stains the ventral commissural axons. Only a single neurite is labelled in the lateral cord at this time of development. Note the absence of labelled processes near the roof plate (RP) in the dorsal spinal cord (200X), (C) Higher magnification of 1C12 labelled neurite in view (B), (arrow), (400X). The roof plate lacks obvious staining with anti-KS antibodies at this stage of development.

Figure 3. The roof plate of E13.5 rat cervical spinal cord. (A) The roof plate (RP) glia extend an apical process to the pial surface, terminating in an endfoot. Interspersed among these glial cells is an extensive network of large extracellular spaces. Together, the cells and spaces form a wedge-shaped region at the dorsal aspect of the spinal cord. Compare the cells of the roof plate with the surrounding region of cells which are closely apposed to one another. The surrounding cells and their processes, some of which are commissural neurons, arch dorsolaterally along the perimeter and then away from the roof plate, (630X). (B) Transverse frozen section of

the same age and cord level as in (A), labelled with an anti-keratan sulfate (a-KS) monoclonal antibody. Keratan sulfate epitopes are specific to the roof plate (RP) at this stage of development. Note that the labelling pattern coincides directly with the wedge-shaped region of glial cells and interspersed extracellular spaces of the roof plate seen in (A); cc, central canal, (630X).

Figure 4. Transverse frozen section of E13.5 rat cervical spinal cord labelled with antibody 1C12. The commissural axons (ca) take a stereotypical path away from the roof plate (RP) as they course from the dorsolateral wall of the spinal cord along the periphery to the floor plate (fp) where they cross the midline and turn to travel in the ventral funiculus. Antibody 1C12 also labels the oval bundle (ob), the dorsal root (dr) and the dorsal root ganglia (drg), (180X).

Figure 5. Transmission electron micrograph of the boundary (arrows) between the roof plate (RP) glia and neighboring neurons and neurites in E13.5 rat cervical spinal cord. No neurites cross the roof plate. An example of one of the large extracellular spaces occurs just below the "RP", (7,000X).

Figure 6. Relationship of the commissural axons to the roof plate (RP) glia in E13.5 rat cervical spinal cord. (A) The commissural axons (ca), localized with monoclonal antibody 1C12, arise from cell bodies (not stained) along the dorsolateral cord and travel away from the roof plate. Commissural axons do not cross the dorsal midline, (250X). (B) An adjacent spinal cord section (low magnification of Fig. 3B) shows the roof plate labelled with an anti-keratan sulfate antibody (a-KS). Note the absence of reaction product anywhere else in the spinal cord. Superimposition of these two views demonstrates the

location of the roof plate glia between, but not overlapping with, the commissural axons; cc, central canal, (250X).

Figure 7. Differential expression of keratan sulfate epitopes in the roof plate of E15.5 rat cervical spinal cord, localized with various anti-keratan sulfate monoclonal antibodies, (A) a-KS, (B) 4-D-1 and (C) 8-C-2 (see Materials and Methods for descriptions). In views (A) and (B), the roof plate is labelled from the pial surface to the central canal, whereas in (C), only the dorsal-most portion of the roof plate is immunoreactive. Further, antibodies 4-D-1 and 8-C-2 recognize epidermis (e), (B and C) and antibody 8-C-2 recognizes the basal lamina (bl) surrounding the cord, (C), (A,B,C, = 400X).

Figure 8. Endo-B-galactosidase and keratanase digestion of the roof plate. (A) An E13.5 rat cervical spinal cord section treated with chondroitinase ABC for over 2 hours at 37° C, then stained for keratan sulfate with antibody (a-KS). The labelling pattern is unchanged from that seen when sections are not pre-treated with chondroitinase ABC (compare to Figure 6B). Skin is also normally stained with this antibody, (400X). (B) E13 rat cervical spinal cord pre-treated with two keratan sulfate-specific enzymes: endo-B-galactosidase and keratanase. Almost no keratan sulfate labelling is observed with anti-KS antibodies following this treatment. These results were duplicated in an E15 animal using antibodies 4-D-1 and 8-C-2, (630X).

Figure 9. Other antibodies and a lectin also recognize the roof plate glia but are not unique to this region. These include (A) L2, (B) 5A5 (highly sialylated N-CAM), (C)a-SSEA-1 and (D) lotus lectin (TPA). Views (A) and (B) are E13.5 and views (C) and (D) are E15.5 rat spinal cord. In (A), compare the roof plate (RP), which is L2 immunoreactive ("V"-shaped pattern) to the floor plate

(fp), which is entirely devoid of reaction product. Just below this clear region lie the decussating commissural axons. This antibody also labels the epidermis (e) and the commissural axons (ca). Antibody 5A5 (B) labels the commissural axons (ca) as well as the roof plate (RP), among others; a-SSEA-1 (C) labels the roof plate (RP) and the floor plate (not shown). Note that this antibody, like 8-C-2 (Fig. 7C) only recognizes the dorsal-most portion of the roof plate. However, some sections show light labelling with this antibody in the lower portion of the roof plate as well. Lotus lectin (D) labels the roof plate (RP) along the dorsal midline; (A, 160X; B, 180X; C, 400X; D, 400X).

Figure 10. Localization of cholinesterase in E13.5 and E15.5 rat cervical spinal cord. (A) The pattern of expression of cholinesterase in E13 spinal cord resembles the pattern of immunostaining for keratan sulfate in the roof plate. Cholinesterase is present in other locations in the cord as well, for example, in the ventricular portion of the basal neuroepithelial cells (be) and the oval bundle of His (ob) (250X). (B) on E15.5, the developmentally regulated change in the roof plate morphology coincides with a change in cholinesterase expression. The pattern of expression of cholinesterase is again similar to that of keratan sulfate. Also stained on E15.5 are the dorsal column axons (dc), the basal epithelial cells (be), the sulcus limitans (sl) and motor neurons (mn), (250X). (C) High magnification of the roof plate shown in (B) demonstrating the close proximity of the dorsal column axons to the roof plate glia at this stage of development. It appears that cholinesterase is localized to a subpopulation of the dorsal column axons, (630X).

Figure 11. Transverse sections of E15.5 rat spinal cord (compare the roof plate with the anti-KS labelled section in Fig. 12). The plastic section shows

the apical and basal processes of the glial cells and their relationship to the pia and central canal (cc). Note the proximity of the dorsal column (dc) axons to the roof plate glia, (630X).

Figure 12. Cryostat sections (10 μm) of rat cervical spinal cord (E15.5) labelled with an anti-KS antibody (a-KS). In (A) note that the dorsal portion of the roof plate is flared out with the ventral portion being thinner; dc, dorsal columns (400X), (B) A similar section at higher magnification, shows the dorsal column axons as they approach the dorsal roof plate cells (arrows) and abut them. As the dorsal column axons continue to enter, they fill the space along the remainder of the roof plate, (650X).

Figure 13. Keratan sulfate epitopes are expressed by other non-innervated regions. Labelling with numerous anti-keratan sulfate antibodies is found (A) and (B) on cells which surround developing rib cartilage in E15.5 rat, and (C) by the outer layer of the epidermis; (A, 250X; B and C, 630X).

Figure 14. The roof plate at E17.5 no longer expresses keratan sulfate epitopes, but labelling is still present in the surrounding cartilage, (150X).

Figure 15. Immunocytochemical labelling of the dorsal midline of optic tectum in hamster mesencephalon with antibodies to keratan sulfate. (A) Labelling with antibody 4-D-1 occurs solely along the tectal midline as shown by horseradish peroxidase reaction product, (400X). (B) The tectal midline is also labelled with antibody 8-C-2 shown here in darkfield with immunofluorescence. Note the intensity of reaction product just above the roof plate in the basal lamina with this antibody, (400X). Other antibodies to keratan sulfate also stain this region (not shown). Note the dense staining at the ventricle.

Figure 16. Substrate preparation technique: 60 mm petri dishes are coated with a mixture of methanol and nitrocellulose and air dried in a laminar flow hood. Cellulose strips (350 μm wide) are soaked in the desired protein solution (e.g. proteoglycan, PG, plus LN or NCAM) + RITC label and transferred to the center of the petri dish in vertical strips (shown by hatched lines). Laminin is then applied to the entire dish with a bent glass pipet followed immediately by media. Dishes are stored in the dark to preserve fluoroescence until DRGs are dissected and ready for seeding. After 24 hours, the plates are fixed, coverslipped and photographed.

Figure 17. Controls. (A) Strips contain 1 μg/ml laminin + RITC and 100 μg/ml laminin is spread over the entire dish. (B) Strips contain 10 μg/ml laminin + RITC and 100 μg/ml laminin is spread over the entire dish. Nitrocellulose only binds the first reagent transferred, thus laminin strips result in alternating concentrations of 1 and 100 μg/ml (A) or 10 and 10 μg/ml (B). Arrows denote boundary of lanes in (A) and RITC fluorescence denotes location of lane in (B). In each case, neurites freely cross the lanes without any signs of inhibition, indicating that neither idiosyncrasies of the protocol nor toxicity of the RITC are problematic in this assay, 250X.

Figure 18. Bovine KS/CS-PG (1 mg/ml) + RITC is transferred in strips with 100 μg/ml laminin spread over the entire dish. Dorsal root ganglia, gently scattered over the center of the dish adhere to the strips of laminin (areas in between strips of KS/CS-PG + RITC) and send out neurites. While abundant growth occurs on laminin, complete inhibition of neurites and support cells occurs when the neurites encounter the KS/CS-PG; 250X.

Figure 19. Neurite outgrowth inhibition by KS/CS-PG is concentration dependent. In this protocol, strips of the proteoglycan were placed from left to right

in increasing concentrations from 0.2 mg/ml to 1.0 mg/ml. This figure shows 0.2 mg/ml (left) and 0.4 mg/ml (center). Arrows denote boundary of lanes. Compare with Figure 18 which shows complete inhibition at 1.0 mg/ml; 40X.

Figure 20. To determine whether neurites were actively inhibited by 1 mg/ml bovine KS/CS-PG or merely stopping due to the lack of a condusive molecule, we mixed laminin with the proteoglycan. In (A), 1.0 mg/ml KS/CS-PG is mixed with 10 μg/ml laminin + RITC (fluorescence shows location of lanes). Neurites are still inhibited by KS/CS-PG, even though a concentration of laminin is present which alone allows for abundant outgrowth (see control in Fig. 17). (B) When the concentration of laminin is raised to 100 μg/ml neurites are able to cross the KS/CS-PG containing strip; 250X.

Figure 21. Response of DRG neurites to a mixture of 1 mg/ml KS/CS-PG with polysialylated NCAM at two concentrations: at 10 μg/ml NCAM, neurites are inhibited by the KS/CS-PG. However, unlike higher concentrations of laminin, 100 μg/ml NCAM is still inhibitory for all but a few neurites (not shown); 40X.

Figure 22. Control for NCAM mixture. Strips containing 10 μg/ml polysialylated NCAM provide a conducive substrate for neurite outgrowth. Neurites growing from 100 μg/ml laminin to NCAM in strips show no pattern change or change in fasciculation; 160X.

Figure 23. Enzyme digestion assay I. (A) DRG neurites are inhibited by 1 mg/ml chick KS/CS-PG in the same manner as seen for bovine KS/CS-PG at the same concentration; vertical arrows denote location of lane boundary; (B) When KS/CS-PG is treated with keratanase, some neurites cross, while many are still inhibited. Vertical arrows denote lane boundary; horizontal arrows point out neurites which have elongated onto the lane; 250X.

Figure 24. Enzyme digestion assay II. (A) Treatment of KS/CS-PG with chondroitin ABC lyase allows many neurites to cross the strips, although some inhibition is still quite evident; vertical arrows denote one of the lanes; 40X. (B) If KS/CS-PG is treated with both keratanase and chondroitin ABC lyase, leaving only the protein core and LN, neurite inhibition is no longer seen. This experiment also serves as a control for the presence of a neutral molecule which shows no inhibitory effect (see Discussion). Arrows and fluorescence denote position of lane; 100X.

Figure 25. A rat chondrosarcoma cartilage proteoglycan (RCS; 1 mg/ml) contains chondroitin sulfate, but not keratan sulfate chains. The chondroitin is in the form of C-4-S and not C-6-S like the bovine and chick KS/CS-PG above. This reagent is not as effective in the inhibition of neurites as is bovine and chick KS/CS-PG, although partial inhibition can be seen. Vertical arrows denote lane boundary; horizontal arrows point out neurites which have elongated onto the lane; 40X.

Figure 26. In vitro assay for C6 glial cell invasion. (A) Inhibition of invasion. No cells are found on the strip. (B) Slight inhibition of invasion. Note the presence of a few cells on the strip, but the cells are not confluent. (C) No inhibition of invasion. Cell migration and confluence are evident on the strip.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the discovery that keratan sulfate (KS), chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin (HN), and/or hyaluronic acid (hyaluronate, HA) can inhibit neurite outgrowth i.e., axonal growth, or nerve regeneration (herein "nerve growth") or glial cell, in particular astrocyte, migration, invasion or regeneration.

Inhibition of neurite outgrowth, i.e., nerve growth, results from KS and/or CS, DS, HS, HN or HA even in the presence of nerve growth promoting factors such as laminin and NCAM. Inhibition of glial cell, in particular astrocyte, migration or invasion results from KS and/or CS, DS, HS, HN OR HA even in the presence of laminin. Accordingly, the present invention is directed to methods of using KS, and molecules and compositions comprising KS to inhibit or prevent neurite outgrowth and/or glial cell migration or invasion, or nerve or glial cell regeneration, and therapeutically, where the foregoing is desired. Such molecules comprising KS include but are not limited to KS glycosaminoglycan and KS proteoglycan, with keratan sulfate proteoglycan most preferred. The invention is further directed to molecules and compositions comprising CS, and the therapeutic uses thereof to inhibit or prevent neurite outgrowth, glial cell migration or invasion, or nerve or glial cell regeneration. Molecules comprising CS include but are not limited to CS glycosaminoglycan and CS proteoglycan, with chondroitin sulfate proteoglycan preferred. The invention is also directed to molecules and compositions comprising dermatan sulfate and therapeutic uses thereof to inhibit or prevent neurite outgrowth, glial cell migration or invasion, or nerve or glial cell regeneration. Molecules comprising DS include but are not limited to DS glycosaminoglycan and DS proteoglycan, with dermatan sulfate proteoglycan preferred. The invention is further directed to molecules comprising heparan sulfate, heparin, and hyaluronate, and therapeutic uses thereof to inhibit or prevent neurite outgrowth, glial cell migration or invasion, or nerve or glial cell regeneration.

In another embodiment, inhibitors and antagonists of KS, CS, DS, HS, HN or HA, and molecules and compositions containing the same, may be used to promote neurite outgrowth or nerve regeneration, i.e., nerve

growth, or glial cell, in particular astrocyte, migration, invasion or regeneration and can be administered therapeutically. Such inhibitors and antagonists include but are not limited to antibodies to KS, CS, DS, HS, HN or HA and derivatives or fragments thereof containing the binding domain, enzymes that degrade KS, CS, DS, HS, HN or HA, lectins specific for KS, CS, DS, HS, HN or HA, and disaccharide antagonists of receptors specific for KS, CS, DS, HS, HN or HA. In this embodiment, promotion of neurite outgrowth, i.e., axonal growth, or glial cell, in particular astrocyte, migration or invasion, or nerve or glial cell regeneration occurs by removing the inhibitory influence of molecules comprising KS, CS, DS, HS, HN or HA, thus allowing promotion of neurite outgrowth, i.e., axonal growth, or glial cell, in particular astrocyte, migration or invasion, or nerve or glial cell regeneration by endogenous or exogenously added molecules.

In a further embodiment of the invention, molecules comprising KS can be used together with molecules comprising another glycosaminoglycan or the disaccharide unit thereof, preferably chondroitin sulfate, in the methods of the invention.

The present invention also provides pharmaceutical compositions comprising effective amounts of molecules and compositions comprising KS, CS, DS, HS, HN and/or HA.

As detailed in the examples sections infra, immunocytochemical localization data is presented which indicates that KS, alone or in combination with other molecules such as chondroitin sulfate, may be in part responsible for the inhibition of axon elongation through the roof plate in the embryonic spinal cord. In a further example, we demonstrate in vitro that keratan sulfate/chondroitin sulfate proteoglycans are actively inhibitory to neurite elongation in a concentration

dependent manner. In other examples, we show that dermatan sulfate inhibits outgrowth of a neuronal cell line and neuronal cells in vitro. A further example in vitro demonstrates that KS/CS-PG and DS-PG inhibit migration and invasion of glial cells and astrocytes.

5.1. THE INHIBITORY COMPOSITIONS OF THE INVENTION

Compositions which are envisioned for use in the present invention to inhibit or prevent neurite outgrowth, or glial cell, in particular astrocyte, migration or invasion, or nerve or glial cell regeneration (termed herein "inhibitory compositions") comprise an effective amount of a molecule consisting of at least the disaccharide unit of KS, CS, DS, HS, HN or HA. Thus, for example, and not as a limitation on the scope of this invention, the molecule can be KS disaccharide, KS glycosaminoglycan, KS proteoglycan, CS disaccharide, CS glycosaminoglycan, CS proteoglycan, DS disaccharide, DS glycosaminoglycan, DS proteoglycan or a compound containing any of the foregoing. In a specific embodiment of the invention, such inhibitory compositions include, in addition to such molecules comprising KS, another glycosaminoglycan or proteoglycan or disaccharide unit thereof, selected from the group consisting of such molecules which comprise chondroitin sulfate (CS) and such molecules which comprise dermatan sulfate (DS). In a particular embodiment, a proteoglycan containing both KS and CS (KS/CS-PG) can be used. Both C-4-S and C-6-S sulfur linkage forms of chondroitin sulfate are envisioned as within the scope of the invention, with the C-6-S form being preferred for the inhibition of neurite outgrowth and nerve growth. The KS for use in the present invention includes but is not limited to Type I (corneal) KS (which is unbranched and highly sulfated, and most easily and completely degraded by endo-b-galactosidase and keratanase

used sequentially (Melrose and Ghosh, 1985, Anal. Biochem. 170:293-300)) and Type II (skeletal) KS. In another particular emodiment of the invention, the molecule may comprise DS.

The molecules comprising KS, CS, DS, HS, HN or HA, and other proteoglycans/glycosaminoglycans for use in the present invention, may be obtained by standard procedures known in the art. For example, KS/CS-PG may be isolated from the cartilage matrix of cell cultures, such as those of limb mesenchymal cells, by published procedures (see Carrino, A. and Caplan, A. I., 1985, J. Biol. Chem. 260:122-127). In another embodiment, KS-PG can be isolated from shark fin, a rich source of KS-PG. In an embodiment where KS disaccharide or KS glycosaminoglycan is desired, KS disaccharide or KS glycosaminoglycan can be isolated after digestion of KS-PG with endo-b-galactosidase or keratanase, respectively (endo-b-galactosidase specifically cleaves between the KS disaccharide residues; keratanase specifically cleaves at the glycosaminoglycan bond to the protein). Alternatively, KS disaccharides and glycosaminoglycans can be chemically synthesized, or purchased from commercial sources.

In brief, and as but one specific example, proteoglycan can be extracted from the cartilage matrix with 4 M guanidinium chloride containing protease inhibitors and purified by CsCl equilibrium density gradient centrifugation and Sepharose CL-2B chromatography (see Haynesworth et al., 1987, J. Biol. Chem. 262:10574-10581).

5.2. THE GROWTH-PROMOTING COMPOSITIONS OF THE INVENTION

The present invention also provides methods of using compositions which promote neurite outgrowth, or glial cell, in particular astrocyte, migration or invasion, or nerve or glial cell regeneration (termed herein

"growth-promoting compositions"). Such growth promoting compositions comprise inhibitors or antagonists or agents which are otherwise destructive (collectively termed herein "growth promoting factors") of the neurite outgrowth and glial cell migration or invasion, or nerve or glial cell regeneration inhibitory activity of keratan sulfate (as exhibited by KS disaccharide, KS glycosaminoglycan, KS proteoglycan, or molecules containing the foregoing). Such growth promoting factors include but are not limited to antibodies which recognize keratan sulfate, and derivatives and fragments thereof which contain the binding domain, enzymes which degrade keratan sulfate, lectins specific for keratan sulfate, and disaccharide antagonists of the keratan sulfate receptor (see Sections 7.1., 6.3. and 7.2.4.4., infra).

In another embodiment, the growth promoting compositions comprise inhibitors or antagonists or agents which are otherwise destructive of neurite outgrowth, or glial cell migration or invasion, or nerve or glial cell regeneration inhibitory activity of CS (as exhibited by CS disaccharide, CS glycosaminoglycan, CS proteoglycan, or molecules containing the foregoing). Thus, the invention is also directed to antibodies to CS (and fragments thereof), enzymes which degrade CS, lectins specific for CS, and disaccharide antagonists of the CS receptor.

In a further embodiment, the growth promoting compositions comprise inhibitors or antagonists or agents which are otherwise destructive of neurite outgrowth, or glial cell migration or invasion, or nerve or glial cell regeneration inhibiting activity of DS (as exhibited by DS disaccharide, DS glycosaminoglycan, DS proteoglycan, or molecules containing the foregoing). Thus, the invention is also directed to antibodies to DS (and fragments thereof), enzymes which degrade DS, lectins specific for DS, and disaccharide antagonists of the DS receptor.

In yet another embodiment, the growth promoting compositions comprise inhibitors or antagonists or agents which are otherwise destructive of neurite outgrowth, or glial cell migration or invasion, or nerve or glial cell regeneration inhibiting activity of HS, HN or HA (as exhibited by HS, HN or HA disaccharide, HS, HN or HA glycosaminoglycan, or HS, HN or HA proteoglycan, or molecules containing the foregoing). Thus the invention is also directed to antibodies to HS, HN or HA (and fragments thereof), enzymes which degrade HS, HN or HA, lectins specific for HS, HN or HA, and disaccharide antagonists of the HS, HN or HA receptor.

5.2.1. ANTIBODY COMPOSITIONS

Antibodies which recognize keratan sulfate, CS, DS, HS, HN or HA, and which may be used, include previously isolated known antibodies as well as antibodies which can be newly generated.

KS disaccharide, KS glycosaminoglycan, KS-PG, or compositions comprising the same, may be used as an immunogen to generate anti-KS antibodies. CS disaccharide, CS glycosaminoglycan, CS-PG, or compositions comprising same, may be used as an immunogen to generate anti-CS antibodies. DS disaccharide, DS glycosaminoglycan, DS-PG, or compositions comprising the same may be used as an immunogen to generate anti-DS antibodies. Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of KS, CS, DS, HS, HN or HA. For the production of antibody, various host animals can be immunized by injection with KS, CS, DS, HS, HN or HA-containing compositions, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin,

pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

In a preferred embodiment, a monoclonal antibody to KS, CS, DS, HS, HN or HA is produced.

For preparation of monoclonal antibodies directed to KS, CS, DS, HS, HN or HA, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the recombinant E. coli library technique of Lerner and colleagues (Sastry et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:5728-5732) and the like are within the scope of the present invention.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851; Takeda et al., 1985, Nature 314:452).

Previously prepared monoclonal antibodies to KS which may be used according to the present invention include but are not limited to antibodies MZ15 (Zanetti et al., 1985, J. Cell Biol. 101:53-59) (specific for sulfated

poly N-acetyllactosamine domains on KS); 1/20/5-D-4 (Caterson et al., 1983, J. Biol. Chem. 258:8848-8854); 4/8/1-B-4 (Caterson et al., 1985, Fed. Proc. 44:386-393) (both 1/20/5-D-4 and 4/8/1-B-4 recognize epitopes that overlap wilth MZ15, but were raised against human articular cartilage and steer nasal cartilage, respectively); 4-D-1; and 8-C-2 (both 4-D-1 and 8-C-2 were generated against chicken bone marrow and recognize two different epitopes of the highly sulfated form of keratan sulfate); and a-Ks Monoclonal antibody 1/20/5-D-4 is commercially available (ICN ImmunoBiologicals, Lisle, Illinois, Cat. No. 696251).

Monoclonal antibody 3-B-3 (commercially available) specifically recognizes the C-4-S form of chondroitin sulfate after digestion of the C-6-5 form by chondroitin ABC lyase (Couchman, J. R., 1984, Nature 307:650-652).

A molecular clone of an antibody to a KS, CS, DS, HS, HN or HA epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoadsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Antibody fragments which contain the binding domain of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide

bridges of the F(ab')2 fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

5.2.2. ENZYME COMPOSITONS

Enzymes that degrade keratan sulfate can be used in the practice of the instant invention, and include but are not limited to endo-b-galactosidase and keratanase. In a specific embodiment, both endo-b-galactosidase and keratanase can be used, simultaneously or sequentially, to degrade KS. In a further embodiment of the invention, enzyme(s) that degrade KS can be used simultaneously or sequentially with enzyme(s) that degrade another proteoglycan/glycosaminoglycan, e.g. enzymes that degrade chondroitin sulfate or dermatan sulfate. In a particular aspect, the enzyme that degrades chondroitin sulfate is chondroitin ABC lyase.

Endo-b-galactosidase, keratanase, and chondroitin ABC lyase are commercially available (e.g., Miles Scientific).

In another aspect, enzymes that degrade CS can be used in the practice of the invention, and include but are not limited to chondroitinase and chondroitin ABC lyase.

In a further aspect, enzymes that degrade DS can be used in the practice of the invention, and include but are not limted to chondroitin ABC lyase.

In yet another aspect of the growth promoting compositions of the invention, enzymes that degrade heparan sulfate, heparin, or hyaluronate can be used. These enzymes include, but are not limited to, heparanase and hyaluronidase.

5.2.3. OTHER COMPOSITIONS

Lectins, also referred to as agglutinins, specific for KS, CS, DS, HS, HN or HA comprise another aspect of the growth promoting compositions of the invention. Lectins that bind to keratan sulfate can be used in the practice of the instant invention. Furthermore, lectins that bind chondroitin sulfate can be used in the practice of the invention. In another aspect, lectins that bind dermatan sulfate can be used in the practice of the invention. In yet another aspect, lectins specific for heparan sulfate, heparin or hyaluronate can be used in the practice of the invention.

In a specific embodiment, the lectin from triticum vulgaris (wheat germ) specific for N-acetyl-D-glircosamine can be used. The triticum vulgaris lectin binds to KS, CS and DS. In another embodiment, the tetrogonolobus purpureas agglutinin (TPA) may be used. Tetragonolobus purpureas is known as asparagus pea, winged pea, and lotus agglutinin or lectin. Other lectins useful in the practice of this invention include, but are by no means limited to, lectins from abrus precatorius (Jequirity bean agglutinin), arachis hypogea (peanut agglutinin), bandeiraea simplicifolia, erythrina corallodendron (Coral tree agglutinin), helix pomatia (Roman snail agglutinin) and helix aspersia (garden snail aglutinin), limulus polyphemus (limulin or horseshoe crab agglutinin), maclura pomifera, (osage orange agglutinin) momordica charantia, phaseolus limensis (lima bean agglutinin), phaseolus vulgaris (red kidney bean agglutinin), psophocarpus tetragonolobus (winged bean agglutinin), sophora japonica (pagoda tree lectin), ulex europas (gorse agglutinin), vicia villosa (hairy vetch agglutinin), vagna radiata (mung bean agglutinin), and wisteria floribunda (Japanese wisteria agglutinin), to name but a few. Any lectin that

binds to the disaccharide, glycosaminoglycan or proteoglycan comprising KS, CS, DS, HS, HN or HA may be used in the growth promoting compositions of the invention.

Disaccharide antagonists that block receptors on nerve or glial cells specific for KS, CS, DS, HS, HN or HA can also be used in the practice of the instant invention. Suitable disaccharide antagonists bind to the receptor for, but do not effect the inhibitory functions of KS, CS, DS, HS, HN or HA.

Metabolic blockers of proteoglycan synthesis may also be used in the practice of the invention. Drugs or agents that inhibit or prevent synthesis or secretion of proteoglycans or glycosaminoglycans prevent the synthesis or secretion of KS, CS, DS, HS, HN or HA, and thus preclude the inhibitory effects of KS, CS, DS, HS, HN or HA.

5.3. THERAPEUTIC USES

5.3.1. THE INHIBITORY COMPOSITIONS OF THE INVENTION

The inhibitory compositions of the invention can be therapeutically useful where an inhibition of neurite outgrowth, glial cell migration or invasion, or nerve or glial cell regeneration is desirable. For example, an inhibitory composition can be used in the treatment of patients with gliomas or tumors of nerve tissue, e.g., malignant tumors such as a neuroblastoma. In another embodiment, an inhibitory composition can be used for the treatment of a neuroma (undirected axon growth associated with situations where the axon is missing either its appropriate target or substrate pathway for neural development). For example, treatment of neuroma associated with amputation, lesion, or congenital deformities, etc. can be treated. Disorders resulting from an overproduction of nerve growth-promoting factors, including but not limited to nerve growth factor, ciliary neurotrophic factor, brain-derived growth factor, laminin, NCAM, L2, and SSEA-1, can also be treated by administration of an

inhibitory composition. The inhibitory compositions can be used to treat disorders of the central and/or peripheral nervous systems.

In another embodiment, the products of this invention can be used as barriers to glial cell migration or invasion caused by trauma, surgery, infection (viral or bacterial), metabolic disease, malignancy, exposure to toxic agents, or other hyperplastic situations. They may be used specifically to protect an organ or tissue from the previously mentioned conditions through a coating procedure. For example, dorsal root ganglia, optic nerve, and optic chiasma may be coated with proteoglycans in order to protect against uncontrolled cell invasion and adhesion. This may be useful as a preventitive or prophylactic treatment or may be applied as a treatment in patients where a disorder has already been manifested.

In one embodiment, compositions including keratan sulfate, in any molecular form in which it may be made or found, either alone or with chondroitin sulfate, which also may be in any molecular form in which it may be found, or dermatan sulfate, which also may be in any molecular form in which it may be found, can be used to preferentially inhibit neurite outgrowth. In another embodiment, keratan sulfate and/or chondroitin sulfate or dermatan sulfate, in any molecular form in which it may be found, may be used to preferentially inhibit glial cell, in particular astrocyte, migration or invasion.

5.3.2. THE GROWTH-PROMOTING COMPOSITIONS OF THE INVENTION

The growth-promoting compositions of the invention can be used therapeutically in regimens where neurite outgrowth is inhibited and an increase in neurite outgrowth or nerve regeneration is desired, e.g., in patients with nerve damage, or in regimens where glial cell, in particular astrocyte, migration, invasion or

regeneration is desired. The growth-promoting compositions can be administered to patients in whom nerves or glial cells have been damaged by trauma, surgery, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, toxic agents, paraneoplastic syndromes, stroke, degenerative disorders of the nervous system, etc. Examples of such disorders include but are not limited to Alzheimer's Disease, Parkinson's Disease, Huntington's chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy, and peripheral neuropathies. In a specific embodiment directed to the treatment of Alzheimer's disease or systemic amyloidoses, in one particular aspect, the growth-promoting compositions can be therapeutically applied so as to allow access to the sites of amyloid plaques (see Selkoe, D. J., 1989, Cell 58:611-612). In another particular embodiment, the growth-promoting compositions of the invention can be used to promote nerve growth through an existing scar or a scar in the process of formation. The growth-promoting compositions may be used in the central and/or peripheral nervous systems, e.g., to prevent the inhibition of and thus promote the regeneration of nerve pathways, fiber systems and tracts.

In a particular embodiment, growth promoting and/or inhibitory compositions of the invention may be used to appropriately direct axon growth along desired paths.

In a further embodiment, the growth promoting compositions of the invention may be used to promote the migration or invasion of glial cells, in particular astrocytes.

5.3.3. PHARMACEUTICAL COMPOSITIONS

The present invention also provides pharmaceutical compositions which comprise an effective amount of an an inhibitory composition or a growth-

promoting composition, as the case may be, and a pharmaceutically acceptable carrier. Such pharmaceutically acceptable carriers include sterile biocompatible pharmaceutical carriers, including, but not limited to, saline, buffered saline, dextrose, and water.

The amount of inhibitory or growth-promoting composition which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In an aspect involving the use of an inhibitory composition, a high concentration of the molecule comprising KS, CS, DS, HS, HN or HA relative to the concentration of factors which promote neurite outgrowth or adhesion (e.g. laminin, NCAM)or glial cell, including astrocyte, migration or invasion at the desired site of therapy, is preferred for use.

5.3.4. MODES OF ADMINISTRATION

Methods of introduction of the pharmaceutical compositions of the invention include methods known to those skilled in the art. It may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Further, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Polymer implants coated

with the pharmaceutical composition can be applied or inserted at the desired site of treatment. Such polymers can have various compositions, pore sizes, and geometries. Polymers which can be used include but are not limited to those made of nitrocellulose, polyanhydrides, and acrylic polymers.

The invention also provides for the pharmaceutical compositions to be administered via liposomes, microparticles, microcapsules, or other semipermeable membranes. In various embodiments of the invention, it may be useful to employ such compositions to achieve sustained release of the inhibitory or growth-promoting compositions.

It is also envisioned that one may introduce cells actively producing an inhibitory or growth-promoting composition into areas in need of such. For example, a recombinant cell secreting an enzyme that degrades KS, CS, or DS can be administered where a growth-promoting composition is indicated. In a different embodiment, a hybridoma cell secreting an anti-KS, anti-CS or anti-DS monoclonal antibody can be administered where a growth-promoting composition is indicated. The cells may be encapsulated in a suitable biological membrane and implanted in the patient.

6. MOLECULAR AND CELLULAR CHARACTERIZATION OF THE GLIAL ROOF PLATE OF THE SPINAL CORD AND OPTIC TECTUM: A ROLE FOR KERATAN SULFATE PROTEOGLYCAN IN THE DEVELOPMENT OF AN AXON BARRIER

Certain types of glial structures, located at strategic positions along the edges of axon pathways, may provide the mechanical and/or chemical elements for the construction of barriers which can grossly direct the elongation of axons during development. The roof plate, a putative axon barrier, is located along the dorsal midline of the developing spinal cord and may be important for the

guidance of the commissural and dorsal column axons. We examined the roof plate to determine the developmental morphology of the region and to determine which molecules were correlated with the barrier function when axons were growing nearby. Light and electron microscopic observations of the roof plate revealed that this glial domain undergoes a dramatic change in shape from a "wedge" with large extracellular spaces between the cell apices at E12.5, to a thin, dense septum with reduced extracellular space at E15.5. Immunocytochemical techniques demonstrated that highly sialylated neural cell adhesion molecule (N-CAM), the carbohydrate recognized by L2 monoclonal antibody, cholinesterase, stage specific embryonic antigen 1, and a ligand that binds tetragonolobus purpureas agglutinin are expressed by the roof plate. These molecules, however, were also found in other regions of the spinal cord which are permissive or attractive to axon growth. A molecule which is unique to the roof plate when axons grow close to, but do not cross, the dorsal midline is a glycosaminoglycan (GAG), keratan sulfate. Keratan sulfate is also present in the tectal midline and in other non-innervated regions such as the outer epidermis and developing cartilage. Our data suggest the possibility that keratan sulfate, alone or in combination with other molecules expressed by the roof plate, may be responsible, in part, for the inhibition of axon elongation through the roof plate in the embryonic spinal cord.

6.1. MATERIALS AND METHODS

6.1.1. PLASTIC SECTIONS AND ELECTRON MICROSCOPY

The trunk region of Sprague-Dawley rat embryos, day 11.5 (E11.5), E12.5, E13.5, E14.5 and E15.5 were fixed by immersion in 4% paraformaldehyde/1% glutaraldehyde in 0.15 M phosphate buffered saline (PBS) overnight at 4°C. The tissue was trimmed, washed in 0.15 M PBS for 1 hour and

post-fixed in 1% osmium tetroxide for 2-3 hours on ice. The sections were washed for an additional hour in 0.15M PBS, dehydrated in ethanol and embedded in Spurr's resin. One micron sections were stained with 1% toluidine blue. Thin sections were stained with uranyl acetate and lead citrate for electron microscopy. In some tissue preparations, the salt concentration of the buffer was varied by 1.5-2.0 to observe the effect on the extracellular spaces between the roof plate glia.

6.1.2. IMMUNOCYTOCHEMISTRY

Embryonic day 11.5, 12.5, 13.5, 15.5 and 17.5 Sprague-Dawley rats were decapitated (except E11.5-12.5 which were small) and fixed by immersion in 4% paraformaldehyde on ice for 2 hours and cryoprotected with 30% sucrose in PBS overnight. Cryostat sections (10-15 μm), collected on gelatin-subbed slides, were incubated in 10 mM containing 1% normal goat serum (NGS) (ICN Immunobiologicals) and 0.1% Triton X-100 (Fisher Scientific Co.), pH 7.2 for 30 minutes at room temperature. Following washing in PBS/NGS, sections were incubated in primary antibody diluted in PBS/NGS/Triton X-100 overnight at 4° C. Sections were washed and incubated with HRP-conjugated goat-anti-mouse IgG or IgM (Boehringer-Mannheim Biochemicals) secondary antibody, or fluorescein-conjugated secondary antibody (Boehringer-Mannhein Biochemicals) overnight at 4° C. For sections treated with the HRP-conjugated secondary antibody, the tissue was then washed and incubated in 3',3'-diaminobenzidine (Sigma Chemical Co.; final concentration = 0.003%) activated with hydrogen peroxide, for approximately 5-10 minutes. The reaction was stopped with buffer and the sections washed with double distilled water, air dried, then dehydrated in increasing concentrations of ethanol and coverslipped in Permount mouting medium (Fisher Scientific Co.). Fluorescein

treated sections were washed with buffer and coverslipped with N-propyl galate to preserve immunofluorescence. Controls did not receive primary antibody, but all subsequent steps were unchanged.

Postnatal day 0 (PO) and P3 Syrian hamster neonates were dissected, decapitated, immersion fixed and cryostat sectioned as described above for the rat embryos, except that sections through the optic tectum were collected instead of spinal cord. The tissue sections were reacted with a series of monoclonal antibodies directed toward a variety of keratan sulfate epitopes described below.

Numerous antibodies to various cell surface and extracellular molecules were tested. Monoclonal antibody 1C12 (Dodd et al., 1988, Neuron, 1:105-116) recognizes a glycoprotein on commissural axons. Antibody 5A5 binds to the polysialic acid moieties on NCAM. It has been shown by others that NCAM may be the only source of polysialic acid in chick brain. Endo-N-treatment of NCAM to remove sialic acid results in a lack of NCAM recognition by antibody 5A5. Further, 5A5 labels a band on a Western blot of brain at 250 kD which corresponds to the molecular weight of highly sialylated NCAM. Anti-SSEA-1 (Solter, D., and Knowles, B. B., 1978, Proc. Natl. Acad. Sci. U.S.A. 75(11):5565-5569) recognizes a stage-specific embryonic antigen which is first expressed in blastomeres of 8-cell stage mouse embryos. The monoclonal antibody MZ15 (Zanetti et al., 1985, J. Cell Biol. 101:53-59), a gift of Ten Feizi of the Clinical Research Center in Harrow, England, has been well characterized using porcine chondrocytes and shown to be highly specific for sulfated poly N-acetyllactosamine domains on keratan sulfate oligosaccharides (Mehmet et al., 1986, Eur. J. Biochem. 157:385-391). Monoclonal antibodies 1/20/5-D-4 (Caterson et al., 1983, J. Biol. Chem. 258:8848-8854) and 4/8/1-B-4 (Caterson et al., 1985, Fed.

Proc. 44:386-393) recognize epitopes that overlap with MZ15, but were raised against human articular cartilage and steer nasal cartilage, respectively. Antibodies 4-D-1 and 8-C-2 were generated against chicken bone marrow and recognize two different epitopes of the highly-sulfated form of keratan sulfate. Monoclonal antibody a-KS is specific to an epitope of keratan sulfate. L2 (Kruse et al., 1984, Nature (London) 311:153-155), a gift from Melitta Schachner (Swiss Federal Institute of Technology, Zurich), recognizes a carbohydrate moiety common to several neural cell adhesion molecules and myelin-associated glycoprotein. Immunocytochemistry for the localization of dermatan sulfate, chondroitin-4- and chondroitin-6-sulfate was used, with chondroitinase digestion (Couchman, J. R., 1984, Nature 307:650-652).

6.1.3. ENZYME DIGESTION ASSAY

Embryonic day 13.5 and 15.5 rats were immersion fixed in 4% paraformaldehyde for 2 hours on ice and cryoprotected in 30% sucrose overnight at 4° C. Cryostat sections (15 lm) were collected on gelatin-subbed slides and air dried. Enzyme treatment consisted of incubating the tissue sections for 20, 40 or 60 minutes with a concentration of 0.001, 0.01 or 0.1 unit/ml of either endo-B-galactosidase or keratanase or both in sequence. Endo-B-galactosidase, isolated from Escherichia freundii, was purchased from Miles Scientific, and was diluted in 0.1 M sodium acetate buffer, pH 5.8. Keratanase, isolated from Pseudomonas sp., was purchased from Miles Scientific and was diluted in Tris hydrochloride buffer, pH 7.4. Controls consisted of: (1) incubating spinal cord sections in chondroitinase ABC enzyme for 20, 40 and 60 minutes at a concentration of 0.1 unit/ml in Tris acetate buffer, pH 7.3, followed by immunostaining for keratan sulfate with antibodies a-KS, 8-C-2 and 4-D-1 according to the above

protocol, (2) incubating a known chondroitin-containing tissue, chick femur (Stage 36) with chondroitinase ABC, keratanase and endo-B-galactosidase, then staining for chondroitin sulfate with antibody 3-B-3 (commercially available) according to the protocol of Couchman, J. R., 1984, Nature 307:650-652. Incubations with chondroitinase were conducted at 37° C and those for keratanase and endo-B-galactosidase were carried out at room temperature.

6.1.4. LECTIN STAINING

Embryonic day 13.5 and E15.5 rats were immersion fixed in paraformaldehyde/glutaraldehyde (either 1:1 or 4:0.1) for 3 hours on ice and cryoprotected in 30% sucrose overnight at 4° C. Cryostat sections (15 lm) of spinal cord were collected on chrom-alum subbed slides. The tissue was then blocked in TBS/BSA (Tris-buffered saline/bovine serum albumin) for 15 minutes at room temperature. The block was removed and the sections were incubated in an HRP conjugate of the winged or asparagus pea lectin, tetragonolobus purpureas agglutinin (TPA), also known as lotus tetragonolobus or lotus lectin (Steindler, D. A., and Cooper, N. G. F., 1987, Dev. Brain Res. 36:27-38) overnight at 4° C. at a concentration of 1:75 or 1:100. TBS/BSA with cations was added to the primary incubation to facilitate TPA binding. The tissue was washed and the TPA visualized with diaminobenzidine (see protocol in Section 6.1.2). Following a final wash, the tissue was dehydrated and coverslipped as above.

6.1.5. CHOLINESTERASE ASSAY

Embryonic day 13.5 and E15.5 rats were immersion fixed in 4% paraformaldehyde in 0.1 M PBS and the spinal cords were cryostat sectioned (10-15 lm). The tissue sections were processed for cholinesterases using a modification of Koelle, G. B., and Friedenwald, J. S.,

1949, Proc. Soc. Exp. Biol. Med. 70:617-622. The sections were rinsed in distilled water numerous times and incubated overnight at room temperature in the dark in a mixture of 0.05 M sodium acetate, 4 mM copper sulfate, 16 mM glycine and acetylthiocholine iodide. The sections were rinsed and incubated in 1% sodium sulfide for 5-10 minutes, rinsed again and incubated in 4% formalin buffer overnight at 4° C. The tissue was rinsed a final time, dehydrated and coverslipped as above.

6.2. RESULTS

The roof plate undergoes morphological changes between embryonic day 11.5 (E11.5) and E12.5. At E11.5, the cells of the roof plate are arranged in an arching pattern in comparison to adjacent neuroepithelial cells which are more radial (Fig. 2A). The extracellular space between the roof plate cells is minimal and comparable to that between the adjacent cells. In comparison, on E13.5, large extracellular spaces, about 2-10 μm in diameter, can be seen between the primitive roof plate glia but not between the adjacent cells (Fig. 3A). The large size and shape of the spaces are consistent in all animals and they are located preferentially along the apical region of the roof plate. At E13.5, the roof plate cells are arranged in the shape of a "wedge". With the electron microscope, we have observed that the apical processes of the roof plate cells terminate at the pial surface in endfeet and the basal processes appear to end at the dorsal central canal. Not every cell in the roof plate spans from the pial surface to the central canal, as these cells are dividing until E14 (Altman, J., and Bayer, S. A., 1984, in Advances in Anatomy, Embryology and Cell Biology, Vol. 85, Springer-Verlag, Heidelberg, Germany, pp. 53-83). The roof plate is approximately 70 μm long from the pia to the central canal at E13.5 and about 100 μm wide at the

midpoint. Rostral-caudal analyses of 1 μm plastic sections indicate that the extracellular spaces are present in the roof plate throughout the cervical and thoracic spinal cord. The spaces appear to be actual and not due to processing of the tissue since various perturbations, e.g. varying the salt concentrations of the buffers by a factor of 1.5-2.0 does not alter the spaces relatively more or less then those in the surrounding tissue. Further, Altman and Bayer (1984, supra) have also observed large caliber extracellular spaces in the roof plate in tissue prepared differently.

Two axon systems are present in the dorsal region of the spinal cord and travel near the roof plate at times which seem appropriate for the roof plate to act as a barrier to them. The first of these, a sub-population of the ventral commissural axons, arise from second-order neurons in the dorso-lateral wall of the spinal cord. The processes of these neurons can be visualized with antibody 1C12 (Dodd et al., 1988, Neuron 1:105-116) (Fig. 4). Neurogenesis occurs in a ventrodorsal gradient and one sees commissural axons arising from lateral cell bodies on E11.5 with very few, if any, axons more dorsally at this time (Fig. 2B and C). However, by E12.5 the commissural axons are located in the dorsal-most cord as evidenced by their 1C12 labelling. The unstained cell bodies of some of the neurons in this group then are located adjacent to the roof plate and their processes arch along parts of the perimeter of the roof plate before turning ventrally. A second neural population, the dorsal column axons, travel very close to the roof plate as they enter the spinal cord and turn in the dorsal funiculus to travel rostrally. On E13.5, these axons reside in the oval bundle about 150 μm from the dorsal midline (Fig. 1). However, by E15 they abut the roof plate at the dorsal midline of the spinal cord. Ultrastructural observations show that neurites do

not cross the roof plate but are found in close apposition to it all along its perimeter (Fig. 5). Thus, it appears that all processes from cells adjacent to the roof plate are excluded from this dorsal midline structure.

In order to characterize the surface or matrix molecules of the roof plate, we examined a variety of antigens and compared their distribution in the roof plate to that in the rest of the spinal cord. Most of the molecules that were expressed by the roof plate were found elsewhere in the spinal cord or in other regions of the embryo (discussed below). However, the distribution of keratan sulfate was restricted solely to the roof plate and was first detectable at about E12.5, but more pronounced at E13.5 (Fig. 3A). Immunocytochemical localization showed that this glycosaminoglycan epitope is present in or on the primitive glial cells. The pattern of expression of this molecule directly coincides with the shape of the roof plate cells and their extracellular spaces revealed in the 1 lm plastic sections of E13.5 embryos (Fig. 3B). Figures 6a and 6B depict the relationship of the keratan sulfate labelling of the roof plate cells to nearby commissural axons labelled with 1C12. Thus keratan sulfate expression appears well before the arrival of the dorsal column axons and seems not to be present prior to, but rather, at about the same time that the dorsal-most commissural population is extending axons. In addition, these markers demonstrate that keratan sulfate epitopes are specific to the roof plate and are found nowhere else in the spinal cord at this stage of development (Fig. 6B). Antibodies a-KS and 4-D-1 label the dorsal midline from the dorsal-most to the ventral-most portion (Figs. 7A and B), while antibodies 8-C-2 (Fig. 7C) and 1-B-4 better recognize the dorsal-most portion. The difference in staining patterns suggests that these antibodies recognize slightly different keratan sulfate epitopes, since keratan sulfate occurs in various

levels of sulfation and chain length (Heinegard, D., and Paulsson, M., 1984, Structure and metabolism of proteoglycans, in K. A. Piez & A. H. Reddi (Ed.), Extracellular Matrix Biochemistry, pp. 277-322, New York: Elsevier Science Publishing Co.), and the roof plate contains a mixture of these epitopes.

Some of the anti-keratan sulfate antibodies also label other structures in the sections we studied. For example, many of the anti-keratan sulfate antibodies label epidermis (Figs. 7B and C), which has been shown by Funderburgh et al. (1986, Dev. Biol. 116:267-277) to contain this glycosaminoglycan. Antibody 8-C-2 additionally labels the basal lamina surrounding the spinal cord (Fig. 7C).

In order to test whether the antibodies that we were using to localize keratan sulfate to the roof plate were specific for this glycosaminoglycan, we used two keratan sulfate-specific enzymes to digest this molecule from the roof plate: endo-B-galactosidase and keratanase. It has been demonstrated by Melrose and Ghosh (1985, Anal. Biochem. 170:293:300) that keratanase alone does not completely digest purified Type I keratan sulfate (corneal KS). However, complete degradation could be achieved when both endo-B-galactosidase and keratanase were used sequentially. They showed that Type II KS (skeletal KS) could not be completely degraded using these two enzymes, but substantial improvement was observed in comparison to the use of either enzyme alone. With the sequential use of endo-B-galactosidase and keratanase in our assay, the intensity of staining in the roof plate, using antibodies specific for keratan sulfate, was largely decreased (Fig. 8B), in comparison to untreated sections as analyzed by eye. Some incubations resulted in imperceptible staining levels, but there was subtle variation from section to section. Other keratan sulfate-containing tissues, such as

cartilage and basal lamina retained much of their keratan sulfate expression following enzymatic digestion, although skin showed some observable decrease in staining intensity. The controls showed that chondroitinase digestion had no visible effect on the intensity of keratan sulfate immunostaining of the roof plate (Fig. 8A) and that keratanase and endo-b-galactosidase had no effect on the staining intensity of chondroitin sulfate antibody on chick femur cells, a tissue known to contain large quantities of chondroitin-6-sulfate (Carrino, A., and Caplan, A. I., 1985, J. Biol. Chem. 260:122-127). Thus, each enzyme appears to be specific for its appropriate substrate. The second control reduces the concern that non-specific proteases contributed to the diminution of labelling in the roof plate by the anti-keratan sulfate antibodies.

The primitive roof plate glia express a number of other characteristic molecules on E13.5, but in contrast to keratan sulfate, these are seen elsewhere in the spinal cord. The carbohydrate recognized by monoclonal antibody L2/HNK-1 (glucuronic acid 3-sulfate) is expressed by the roof plate cells (Fig. 9A). In contrast, the floor plate is entirely devoid of labelling with L2. Antibody 5A5 localizes highly sialylated NCAM to only the midline portion of the roof plate (Fig. 9B). Both L2 and 5A5 label the DRG, the dorsal and ventral roots, the dorsal root entry zone and the entire marginal zone of the spinal cord. A histochemical assay for cholinesterase (ChE) showed that the roof plate glia express this molecule as well on E12.5 and E13.5 (Fig. 10A). The pattern of ChE expression on E12.5 and E13.5 in the roof plate is like that of the anti-keratan sulfate antibodies at this age, i.e. in a wedge-shaped distribution. ChE staining is also present in the dorsal root entry zone, on glial cells of the sulcus limitans and the ventricular portion of the basal plate neuroepithelia at this time (Fig. 10A).

The roof plate undergoes a second and more dramatic morphological alteration by E15.5. The presumptive glial cells become transformed into a long, thin septum-like structure in the dorsal midline (Fig. 11). The extracellular spaces are greatly diminished, resulting in a denser construct than that seen before this age. The roof plate spans approximately 160 μm from the pial surface to the top of the central canal, but is only 10-15 μm wide, except at its dorsal aspect where it widens. Thus, the roof plate has undergone about a two-fold increase in length and approximately a ten-fold decrease in width.

Developmental changes occur in the distribution of keratan sulfate epitopes as well as E15.5 and their localization reflects the morphological pattern change described above (Fig. 12A). With the use of differential interference contrast (DIC) microscopy, one can appreciate that the medial-most dorsal column axons (arrows) abut the flared out, dorsal portion of the keratan sulfate-labelled roof plate cells (Fig. 12A and B).

Keratan sulfate epitopes were expressed by developing cartilage. Label is present surrounding groups of chondrocytes (Fig. 13) and in some cases around the individual chondrocytes themselves.

Other markers expressed by the roof plate also change their distribution during development. On E15.5, the roof plate no longer demonstrates positive labelling with antibodies L2/HNK-1 or 5A5, but does become SSEA-1 positive (Fig. 9C). The expression of this molecule resembles that of 8-C-2 and 1-B-4 antigens in that it is present in or on the dorsal-most but not the ventral-most portion of the roof plate glial cells. SSEA-1 antigen is not unique to the roof plate. It is expressed by the floor plate glia as well. The lectin TPA also binds to the roof plate glia and many other radial cells on E15.5. TPA labels the doral midline in its entirety from the pial

surface to the dorsal central canal (Fig. 9D). ChE is present along the dorsal midline from the pial surface to the top of the central canal on E15.5 (Fig. 10B and C). At this stage, it is also expressed by a subpopulation of sensory axons, in the ventricular portion of the basal neuroepithelia, the motor cells, the ventral root and in the sulcus limitans, as well as in the developing limb bud cartilage of the upper trunk.

By E17.5, the roof plate occupies a minimal area of the dorsal midline laterally but still spans from the pial surface to the dorsal central canal. Keratan sulfate epitopes are no longer detectable with immunocytochemistry in the roof plate (Fig. 14) nor in the basal lamina surrounding the spinal cord at E17.5, but they persist in cartilage and epidermis.

In an effort to find any similarity between the roof plate and other putative dorsal midline axon barriers in the central nervous system, we tested normal hamster optic tectum for the presence of keratan sulfate. The hamster was chosen in view of work by Schneider (Schneider, G. E., 1973, Brain Behavioral Evolution 8:73-109) and Poston et al. (Poston et al., 1988, Society for Neuroscience Abstract 14:594) which has shown that neonatal lesions that are focused on the tectal midline allow for the abnormal development of a recrossed optic projection (see Discussion). Indeed, the dorsal midline of the hamster tectum is keratan sulfate positive with antibodies 4-D-1 (Fig. 15A), 8-C-2 (Fig. 15B) and 1-B-4 (not shown) on the first day of birth (PO) and on P3, a time when this midline region could function normally to disallow optic axons from passing between the tecta. As in the rat spinal cord, keratan sulfate labels solely the midline of the mesencephalon. Labelling can be seen along the entirety of the dorsal midline from the pial surface to the dorsal cerebral aquaduct of Sylvius.

6.3. DISCUSSION

We have shown that the roof plate of the spinal cord undergoes morphological and molecular changes during early embryonic development. On E13.5, a network of large extracellular spaces develops near the pial surface between the glial cells of the roof plate and contributes to the roof plate's wedge shape. By E15.5, the shape of the roof plate has changed to a long, thin septum at the midline and the amount of extracellular space is significantly reduced. Although the roof plate cells express a number of molecules which are also present in other regions of the spinal cord, a particular glycosaminoglycan, keratan sulfate, is expressed solely by the roof plate glia beginning on E12.5 and is no longer detectable by E17.5. By following the pattern of keratan sulfate staining in relation to developing fiber systems in the spinal cord, we have observed that the roof plate is never invaded by axons when keratan sulfate is present and that it eventually becomes the midline boundary of the dorsal-most portion of the ventral commissural pathway and of the dorsal columns. The dorsal gray commissure, which crosses the dorsal midline at about E17.5 (Smith, C. L., 1983, J. Comp. Neurol. 185:1-22), develops at a time when keratan sulfate is no longer detectable with immunocytochemistry. It is likely that the roof plate interacts specifically with, exerting its inhibitory influence during development on, axons that elongate near the midline, i.e. a small subpopulation of the ventral commissural system and a larger number of axons which constitute the medial-most (i.e. gracile tract) fibers of the dorsal columns.

It is likely that keratan sulfate does not occur alone in vivo but rather as a keratan sulfate/chondroitin sulfate proteoglycan (KS/CS-PG). We did not find that the roof plate glia express chondroitin sulfate using immunocytochemical techniques, but this could be due to the

fact that glycosaminoglycans can be difficult to fix or stain. It is possible that chondroitin sulfate as well as other glycosaminoglycans and/or proteoglycans may be present in the roof plate and may be acting in combination with other molecules such as keratan sulfate glycosaminoglycan/proteoglycan to generate axon inhibition.

6.3.1. GLIAL CELLS AS AXON BOUNDARIES AND BARRIERS

It has been suggested that glial structures may act as axon barriers or boundaries in regions of the nervous system other than the roof plate. A glial structure at the diencephalic/telencephalic junction near the front edge of the optic chiasm in mouse (Silver, J., 1984, supra) and chick (Silver et al., 1987, supra; Poston et al., 1985, supra) appears to act as a barrier to developing optic fibers. The "knot-like" structure, which is suggested (Silver, J., 1984, supra) to be comprised of the progenitor cells of the 02A lineage (Raff et al., 1983, Nature, 303:390-396), may ensure that the migrating fibers choose one of the functionally advantageous pathways toward the midbrain and diencephalon instead of turning rostrally to enter the olfactory region of the telencephalon.

Studies on the developing optic tectum in the Syrian hamster have revealed that a dorsal midline barrier comprised of glial cells may serve an important function for establishing normal axon-to-target connections. Classic studies of Schneider, G. E., 1973, supra and So, K., 1979, Journal of Comparative Neurology, 186:241-258 have shown that early unilateral lesions of the optic tectum (superior colliculus) result in aberrant crossing of the optic tract axons and functionally maladaptive synaptic connections within the inappropriate tectal lobe. Recently, we have learned that crossing occurs if damage is focused on the midline alone and the dorsal basal lamina remains intact (Poston et al., 1988, Society for

Neuroscience Abstract 14:594). Interestingly, this tectal midline boundary consists of glial cells which are much like those of the roof plate of the spinal cord. They maintain a primitive radial morphology and also express keratan sulfate specifically during development. This suggests that the dorsal midline of the mesencephalon may play an important role in the normal maintenance of side restriction for migrating optic axons and that a glial structure can act functionally as a barrier to growth cones. If the roof plate of the developing spinal cord functions similarly to the midline glial structure of the tectum, it may constitute an essential blockade to aberrant axon elongation. In general, barriers at the dorsal midline of the central nervous system may be instrumental in separating right versus left side sensory information.

Glia may also serve to compartmentalize regions of axonal arborization. A type of boundary glia identified by anti-glial fibrillary acidic protein (GFAP) has been observed in neonatal cortex (Cooper, N. G. F., and Steindler, D. A., 1986, Brain Res. 380:341-348). The parcellation by these cells of the somatosensory barrel fields of cortical Layer IV in mouse correlated directly with patterning of the mystacial vibrissae. The glia are delineated by their dense expression of glycoconjugates that are specifically recognized by certain lectins (Steindler, D. A., and Cooper, N. G. F., 1987, Dev. Brain Res. 36:27-38). Glial cells of the barrel wall domains apparently reflect the mature patterning of the thalamic terminal arbors related to vibrissal function. However, this form of boundary differs from the roof plate. It appears to be more plastic since the intense matrix producing-glia of the barrel walls are able to shift their position geometrically in response to an activity-dependent signal associated with the afferent axons. Cells that may play a similar role in cordoning synaptic territories have

also been observed by Oland et al. (1988, J. Neurosci. 8(1):353-367) in the olfactory region of the moth, Manduca sexta.

The roof plate cells of the spinal cord deserve discussion on three separate but interrelated aspects which may provide evidence about their shape and mechanism of axon repulsion: (1) the possible structural contribution of the extracellular space between the glial cells, (2) the absence of growth of axons through the extracellular spaces and (3) the inhibitory functions of the molecules expressed by these cells.

6.3.2. EXTRACELLULAR SPACE IN THE ROOF PLATE

Developmental changes in the amount of extracellular space may be important for creating the shape and density of the roof plate. At E13.5, it seems likely that the placement of the extracellular spaces, preferentially along the apical region of the roof plate, could play a role in the construction of a wedge (Fig. 3A). By E14.5, since the cells are no longer dividing (Altman, J., and Bayer, S. A., 1984, in Advances in Anatomy, Embryology and Cell Biology, Vol. 85, Springer-Verlag, Heidelberg, Germany, pp. 53-83) and the spinal cord is expanding laterally, the extracellular spaces may be contributing to or allowing for this expansion. The keratan sulfate and probably also the chondroitin sulfate chains of the KS/CS-PG could possibly help play a role in the creation of the extracellular spaces since it is known that glycosaminoglycans in general bind water preferentially (Margolis et al., 1975, Biochem. 14(1):85-88).

6.3.3. AXON OUTGROWTH IN RELATION TO THE ROOF PLATE

It was somewhat surprising that axons do not grow in the roof plate, since in this (Altman, J., and Bayer, S. A., 1984, supra) and other developing neural systems, it has been suggested that large, matrix-filled extracellular spaces between glial cells, when aligned into channels, may act in a permissive or instructive manner to guide axons toward their targets (Silver, J., and Robb, R. M., 1979, Dev. Biol. 68:175-190; Singer, et al., 1979, J. Comp. Neurol. 185:1-22; Silver, J., and Sidman, R. L., 1980, J. Comp. Neurol. 189:101-111; Nordlander, R. and Singer, M., 1982, Exp. Neurol. 75:221-228). Evidence exists that the migration of neural crest cells is guided by their channel-like extracellular environment and that the extracellular matrix is directly involved (Bronner, M. E., and Cohen, A. M., 1979, Proc. Natl. Acad. Sci. USA 76:1843-1847; Bronner-Fraser, M., and Cohen, A. M., 1980, Dev. Biol. 77:130-141; Erickson et al., 1980, Dev. Biol. 77:142-156; LeLievre et al., 1980, Dev. Biol. 77:362-378; Bronner-Fraser, M., 1982, Dev. Biol. 91:50-63). However, our observations of the roof plate show that the mere physical presence of large extracellular spaces is not necessarily predictive of a future axon pathway, since the spaces in the dorsal midline define a region which appears to be refractory to axon growth. Our data indicates that keratan sulfate glycosaminoglycans/proteoglycan, alone or in combination with other glycosaminoglycans/proteoglycans, in the spaces or perhaps on the surface of the glial cells, extending into the spaces, may function to modify the environment in order to generate the refractory characteristics of a barrier.

6.3.4. MOLECULES EXPRESSED BY THE ROOF PLATE GLIA

Keratan sulfate epitopes may function, in part, in the creation of a molecular barrier in the roof plate. Also, we have observed with light microscopy that the region surrounding developing cartilage and the matrix around individual chondrocytes express keratan sulfate-like immunoreactivity. Cartilage is not innervated. Further, we have shown that outer epidermis expresses keratan sulfate epitopes during development.

Importantly, tissue culture experiments demonstrate that keratan sulfate glycosaminoglycans can directly inhibit axon growth (see Section 7 infra).

An obvious consideration is whether the antibodies we used were recognizing keratan sulfate in the roof plate or rather were binding to another antigen which is keratan sulfate-like in molecular composition. A factor in favor of specificity was that so many different well-characterized antibodies to various keratan sulfate epitopes stain the roof plate. Strong evidence, however, resulted from our enzyme degradation studies. The fact that incubation of spinal cord sections with two keratan sulfate specific enzymes (while controlling for non-specific effects) reduces the intensity of antibody staining suggests: (1) that keratan sulfate is indeed expressed on or in the roof plate glia, and (2) that the roof plate may contain Type I (corneal) KS. Corneal keratan sulfate is a molecule which is unbranched and highly sulfated and one that is most easily and completely degraded by endo-B-galactosidase and keratanase used sequentially (Melrose and Ghosh, 1985, Anal. Biochem. 170:293-300).

Our present studies indicate that there are molecular differences between the dorsal-most and ventral-most roof plate since antibodies anti-SSEA-1, 1-B-4 and 8-C-2 label only the dorsal portion. This heterogeneity may

be important with respect to the temporal and regional variation in axon inhibition in the roof plate, since, at late stages, the dorsal commissure develops through the central portion of the dorsal midline (Smith, 1983, J. Comp. Neurol. 220:29-43). Importantly, the formation of the commissure occurs near the time when the expression of a keratan sulfate diminishes below detectable levels.

The presence of cholinesterase in a putative axon barrier region, the roof plate and in cartilage, along with evidence from the above studies, suggest that cholinesterase could potentially influence neurite outgrowth.

While L2, sialylated NCAM and SSEA-1 have been implicated in cell-cell adhesion, their possible role in the roof plate may be to generate morphogenetic density which appears to increase between the roof plate glia during development. Lacking any other influences, these molecules could potentially attract axons into the roof plate. A negative or inhibitory factor may be required to override this potentially axon-attractive milieu.

7. KERATAN SULFATE/CHONDROITIN SULFATE PROTEOGLYCAN (KS/CS-PG) INHIBITS NEURITE OUTGROWTH IN VITRO

As detailed in Examples Section 6 herein, in vivo studies of the roof plate and optic tectum in rodent and the developing subplate in the telencephalon of the chick showed that two glycosaminoglycans, keratan sulfate, alone or in combination with chondroitin sulfate, possibly in the proteoglycan form (KS-PG, CS-PG, or KS/CS-PG) were present within these regions at times when axons approach closely but do not invade these territories. In order to determine if KS-CS/PG actively inhibits growth cone elongation and to determine which component(s) of this macromolecule may be critical to this phenomenon, we used a technique employing nitrocellulose-coated petri dishes onto

which strips of various purified molecules were transferred. We grew E9 chick dorsal root ganglia on lanes of KS/CS-PG in alternation with lanes of the growth-promoting molecules laminin (LN) or NCAM. Neurites grew abundantly along stripes of LN or NCAM. In contrast, upon encountering a stripe containing KS/CS-PG, neurites either stopped abruptly or turned and travelled along the KS/CS-PG strip border in a concentration dependent manner. To determine whether the inhibitory effect was due to the presence of KS/CS-PG or merely to the absence of LN or NCAM in the axon-free lane, we mixed LN or NCAM with the KS/CS-PG, in concentrations which alone support luxurious outgrowth, and observed that the KS/CS-PG was still inhibitory when the attractive molecules were present. KS/CS-PG plus NCAM remained inhibitory even at very high concentrations of NCAM. However, high concentrations of LN were able to overcome the inhibitory effect of the KS/CS-PG. Enzymatic digestion of the KS or CS from the KS/CS-PG permitted various degrees of neurite outgrowth to occur across the previously inhibitory lanes, and digestion of both glycosaminoglycan moieties, leaving only the protein core of the molecule, resulted in a complete lack of inhibition. A proteoglycan containing a different glycosaminoglycan, dermatan sulfate (DS-PG), and a rat chondrosarcoma cartilage CS-PG, were not as effective in axon inhibition as KS/CS-PG at the same concentrations. These assays demonstrated that KS/CS-PG is actively inhibitory to embryonic dorsal root ganglia neurites in vitro. Complete inhibition required contributions from both KS and CS moieties.

7.1. MATERIALS AND METHODS

7.1.1. SUBSTRATE PREPARATION

Tissue culture substrates were prepared by coating 60-mm Petri dishes evenly with 0.5 ml of a mixture of 5 cm2 nitrocellulose (Schleicher & Schuell, Type BA 85) dissolved in 6 ml methanol and allowing them to air dry in a laminar flow hood (Lagenaur, C. and Lemmon, V., 1987, Proc. Natl. Acad. Sci. USA 84:7753-7757). Cellulose (Whatman Filter paper, #1) was cut into 350 μm strips and used to blot various protein substances down onto the nitrocellulose substrate. Each protein solution contained either rhodamine isothiocyanate (RITC) or fluorescein isothiocyanate (FITC) as a marker which could later be detected to determine the exact position of the strips. The cellulose strips were soaked in 20 μl of the desired protein solution, transferred to the nitrocellulose-coated dish in a vertical pattern (FIG. 1), allowed to set for 30 seconds then removed. Following drying of the test molecules onto the nitrocellulose, a thin coat of 100 μg/ml laminin (LN) (Gibco, Inc.) was spread evenly across the dish with a bent glass Pasteur pipette. Media was immediately added to the dish (DMEM/F12 + 10% fetal calf serum + 5% chick embryo extract + 1% antibiotics), and the dish was stored in an incubator until needed. Importantly, the experiments were repeated using serum-free media, DMEM/F12 + N2 (1:100, Bottenstein, J. E. and Sato, G. H., 1979, Proc. Natl. Acad. Sci. USA 76:514-517) + 1% antibiotics, since it is known that serum may give inaccurate results with respect to its effect on proteoglycan binding. In our hands, however, the presence or absence of serum in the media was not critical to the results of the assays.

7.1.2. DORSAL ROOT GANGLIA PREPARATIONS

Chick E9 dorsal root ganglia (DRG) were dissected in a calcium-magnesium free buffer by decapitating the chick, eviscerating, then carefully removing the vertebral column and spinal cord. The DRGs were then cleaned free of surrounding tissue and plucked out using fine forceps. The media in the test culture dishes was removed and replaced with fresh media containing 100 ng/ml nerve growth factor. With a finely drawn glass Pasteur pipette, the DRGs were picked up and scattered gently around the center of the dish containing the patterned stripes. Approximately 20 DRGs were seeded onto each dish. The dishes were then incubated for 24 hours followed by fixation with 4% paraformaldehyde/0.1% glutaraldehyde for 1 hour. The dishes were coverslipped in Mowial and and observed with a Leitz Orthoplan 2 fluorescent microscope, equipped with a Variolume which allows one to mix phase optics with fluorescence so as to observe the neurites and the location of the stripes simultaneously. Each experiment was repeated at least 3 times.

7.1.3. DOT BLOT IMMUNOASSAY

To assure ourselves that the KS/CS-PG was being bound to the nitrocellulose, we conducted a dot blot immunoassay using various anti-keratan sulfate antibodies (an alternative method was to use 35S-labelled proteoglycan; see below). Small squares of nitrocellulose paper were placed into 4-chambered dishes. Each chamber was spotted with 1 μl of the KS/CS-PG and air dried for 5 minutes. The paper was then blocked with straight normal goat serum (NGS) for 10 minutes then washed 5X with buffer. Each well was filled with a different anti-keratan sulfate antibody: 8-C-2 and 4-D-1, a-KS, 5-D-4 and 1-B-4 (Caterson et al., 1985, Fed. Proc. 44:386-393) or MZ15 (Zanetti, et

al., 1985, J. Cell Biol. 101:53-59), 1:100 in a mixture of 10 mM PBS + 3% NGS + 0.2% Triton X-100 and incubated at 37°C overnight. The wells were rinsed 5X with buffer, and a goat anti-mouse HRP-conjugated IgG or IgM secondary antibody was added and incubated overnight at 37°C. The nitrocellulose paper was then reacted with 0.01% diaminobenzidine in PBS + 0.003% hydrogen peroxide. All dots showed a reaction product indicating that the KS/CS-PG was bound to the paper.

7.1.4. ^{35}S-LABELLING OF KS/CS-PG

To determine the amount of proteoglycan which binds to the culture substrate, labelled material was used to coat the substrate by the same procedure as that used for the DRG cultures. Labelled proteoglycan was originally isolated from the cartilage matrix of day 8 chick limb mesenchymal cell cultures after the cultures were labelled with 50 μCi/ml [^{35}S]-sulfate (DeLuca et al., 1977, J. Biol. Chem. 252:6600-6608). The proteoglycans were extracted with 4 M guanidinium chloride containing protease inhibitors and purified by CsCl equilibrium density gradient centrifugation and Sepharose CL-2B chromatography (Haynesworth et al., 1987, J. Biol. Chem. 262:10574-10581). The appropriate fractions from the Sepharose CL-2B column were pooled, dialyzed against distilled water at 4°C and lyophilized to dryness so that the number of ^{35}S cpm/mg dry weight could be determined. After the labelled proteoglycan was bound to the culture substrate, the area of the culture dish containing the bound proteoglycans was excised and transferred to a 20-ml glass scintillation vial. Cytoscint scintillation cocktail (ICN) was added to the vial and the amount of bound ^{35}S was determined by scintillation spectrometry on a Beckman LS 6800 counter.

From the amount of bound 35S, the amount of bound proteoglycan could be calculated based on the number of 35S cpm/mg dry weight.

7.1.5. IMMUNOCYTOCHEMISTRY FOR THE DETECTION OF LAMININ

To detect the presence of laminin in the strips, we used a polyclonal anti-laminin antibody (BRC, V. Lemmon) to label strips of the KS/CS-PG + LN. The 60 mm nitrocellulose-coated dishes were blotted with bovine KS/CS-PG (1 mg/ml) + LN (10 μg/ml). To block non-specific binding, we incubated the strips in 10 mM PBS with 10% normal goat serum (PBS/NGS) for 30 minutes at room temperature. The dish was then incubated with the primary antibody in PBS/NGS, washed 5X with buffer, incubated another hour with a goat anti-rabbit IgG conjugated with FITC, washed 5X with buffer and coverslipped with N-propyl galate to preserve fluorescence. The presence of LN was easily visualized in strips which mimicked exactly the lanes formed originally with the LN/PG mixture.

7.1.6. MODIFICATIONS OF THE DORSAL ROOT GANGLIA ASSAY

7.1.6.1. KS/CS-PG:LAMININ MIXTURES

We made stripes on nitrocellulose plates which were strips containing mixtures of laminin and the KS/CS-PG, varying the laminin concentration among four plates: 100 μg/ml, 50 μg/ml, 25 μg/ml, and 10 μg/ml. The concentration of the KS/CS-PG was maintained at 1 mg/ml, which we knew to produce maximum inhibition of neurites. The remainder of the experiment proceeded as above. Controls consisted of strips of 10 μg/ml laminin + RITC and 10 μg/ml laminin spread over the whole dish (in the basic experiment 100 μg/ml was used), followed by seeding of DRGs and 24 hours incubation to determine whether this concentration of laminin is sufficient for normal outgrowth.

7.1.6.2. KS/CS-PG:NCAM MIXTURES

Polysialylated NCAM is present in the roof plate during development (see Section 6, supra). In order to test the effect of a combination of the proteoglycan with NCAM, we mixed the PG and NCAM (gift of P. Yang and U. Rutishauser) in lanes in alternation with LN. Polysialylated NCAM was prepared by immunoaffinity purification in milligram quantities from the 0.5% NP40 extracts of E14 chick brain vesicles. NCAM in the extracts binds to anti-chick NCAM monoclonal antibody (5E) IgG conjugated to Sepharose 4B beads which are activated by the cyanogen bromide method, and NCAM is eluted with 0.57% diethylamine, pH 11.5 (Hoffman et al., 1982, J. Biol. Chem. 257(13):7720-7729). The resulting NCAM is polysialylated and runs above 200 kD on SDS polyacrylamide gels. NCAM (10 or 100 μg/ml) was used in combination with 1 mg/ml KS/CS-PG and the incubations conducted as above. These dishes were compared to the KS/CS-PG + LN mixtures and to the KS/CS-PG alone.

7.1.6.3. ENZYME DIGESTION OF KERATAN SULFATE CHAINS

Two degrading enzymes specific for keratan sulfate chains, endo-B-galactosidase and keratanase (Melrose, J., and Ghosh, P., 1985, Analytical Biochemistry, 170:293-300) (Miles Sci.), and/or an enzyme specific for chondroitin sulfate chains, chondroitin ABC lyase (Miles Sci.), were added at a concentration of 100 U/ml to dishes with stripes that contained a mixture of laminin (10 μg/ml) and bovine or chick KS/CS-PG (1 mg/ml). With respect to endo-B-galactosidase and keratanase, this concentration was observed to significantly degrade the keratan sulfate in 10 lm frozen sections of rat spinal cord. The DRGs were seeded and the cells were incubated for 24 hours as done previously. Dishes containing the enzyme treated stripes were incubated simultaneously with control dishes which did

not receive enzyme and neurite outgrowth was compared. Certain control dishes included protease inhibitors (1 mg/ml each of apoprotin, leupeptin and pepstatin in 10 mM Tris-acetate buffer, pH 7.2).

Further enzyme digestion products were prepared in solution starting with chick KS/CS-PG, originally isolated from day 8 chick cartilage limb mesenchymal cell cultures (Carrino, A., and Caplan, A. I., 1985, J. Biol. Chem. 260:122-127), as follows: (1) keratanase only to remove keratan sulfate chains from the proteoglycan, (2) chondroitin ABC lyase only to remove chondroitin chains, and (3) a combination of both keratanase and chondroitin ABC lyase to produce a pure protein core. Immuno dot blots showed that all of the chondroitin chains were removed by this treatment, but approximately 10% of the keratan sulfate chains remained attached to the protein core after digestion. The protein core + LN dishes served as a control to test for the possible effect of mass action. All were reacted at 37°C for 1 hour. Each of the four reagents, mixed with LN, were assayed for their inhibitory effect on E9 chick DRG neurites as done previously.

7.1.6.4. RAT CHONDROSARCOMA CARTILAGE PROTEOGLYCAN

A chondrosarcoma cartilage proteoglycan (RCS) (Carrino and Caplan, 1985, J. Biol. Chem. 260:122-127) was used in the basic assay. This proteoglycan is much like the bovine or chick KS/CS-PG except that it lacks the KS chain region and the chondroitin sulfate is in the C-4-S form rather than the C-6-S form found in bovine and chick KS/CS-PG.

7.1.6.5. DERMATAN SULFATE PROTEOGLYCAN ASSAY

In order to find out if neurite inhibition with bovine and chick KS/CS-PG was common to other proteoglycans, we used dermatan sulfate proteoglycan (DS-

PG), as one of many other possible glycosaminoglycans, in the basic protocol described above. The DS-PG was used as before at the same concentration and mixed with the laminin, as previously done with chick and bovine KS/CS-PG. Chick (E9) DRG neurons were seeded on the test culture and analyzed.

7.2. RESULTS

Our goal was to develop an assay which could demonstrate whether keratan sulfate/chondroitin sulfate proteoglycan (KS/CS-PG), alone or in combination with other molecules present in or around the roof plate, could actively inhibit neurite outgrowth in vitro. To do this, we used modifications of a cell culture technique developed by Lagenaur and Lemmon, (1987, Proc. Natl. Acad. Sci. USA 84:7753-7757) which utilizes nitrocellulose-coated petri dishes onto which purified molecules can be applied in specific geometric patterns.

7.2.1. ASSAY FOR INHIBITION OF NEURITE OUTGROWTH

Nitrocellulose is a substrate easily adhered to petri dish plastic, which allows for the noncovalent attachment of proteins for culture studies. For this reason, we employed nitrocellulose-coated culture dishes as a substrate by which to attach such proteins as laminin (LN) and neural cell adhesion molecule (NCAM), which are known to facilitate cell attachment and/or allow for the elongation of neurites (Rutishauser et al., 1978, J. Cell Biol. 79:382-393; Letourneau, P. C., 1975, Dev. Biol. 44:92-101; Manthorpe et al., 1983, J. Cell Biol. 97:1882-1890; Liesi et al., 1984, J. Neurosci. Res. 11:241-251; Lander et al., 1985, Proc. Natl. Acad. Sci. USA 82:2183-2187; Cohen et al., 1986, Nature 322:465-467; Mirsky et al., 1986, J. Neurocytol. 15(6):799-815, and present results). We also attached the proteoglycans of interest,

i.e. keratan sulfate/chondroitin sulfate proteoglycan (KS/CS-PG) or various portions of this macromolecule (its protein core, or KS-PG, or CS-PG). In addition, we bound dermatan sulfate proteoglycan (DS-PG) and a rat chondrosarcoma cartilage proteoglycan (RCS) for comparison. Whole chick (E9) dorsal root ganglia (DRGs) were seeded onto the culture dishes which contained vertical stripes of the purified molecules (Fig. 16). The assays were conducted in serum-containing media and then repeated in serum-free media for comparison. The results of each assay were unchanged regardless of the presence of serum. Analysis of neurite outgrowth was conducted by separating our observations of those DRGs which adhered to the LN-coated surfaces away from the PG lanes from those that set partially or fully on the proteoglycan-containing stripes. It was not possible to quantitate exactly the number of inhibited neurites in comparison to those that were not inhibited, i.e. those that crossed the PG lanes, because the variable fascicle thicknesses and the anastomotic nature of the neurites prevented an accurate count. In all cases considered, the result was obvious as to whether the bundle of neurites was completely inhibited or not and was considered in a "yes-or-no manner" with respect to inhibition. When intermediate patterns resulted, a representative photo was taken of the average case.

7.2.2. THE EFFECT OF GROWTH-PROMOTING MOLECULES

Test cultures consisting of RITC-labelled stripes of 1-100 μg/ml LN alone supported neurite outgrowth from the E9 chick DRGs. Culture dishes with 1 or 10 μg/ml LN strips placed centrally with RITC added and with 10 or 100 μg/ml LN spread over the entire dish stimulated the production of a symmetric halo of neurites around each DRG with no difference in growth at the lines between the LN/RITC laid down in stripes and the LN spread over the

dish to occupy the remaining stripes (Figs. 17A and B). This control demonstrates that technical idiosyncrasies of the culture set-up or toxicity problems from labelling the stripes with RITC are not a factor in this paradigm. Controls with DRGs grown on nitrocellulose alone showed little or no cell attachment or neurite outgrowth.

7.2.3. THE ROLE OF INHIBITORY MOLECULES

When KS/CS-PG labelled with RITC was blotted in stripes onto the nitrocellulose-coated culture dish and LN coated over the entire dish to occupy the remaining lanes, the neurites extending from the DRGs either stopped at the border of the KS/CS-PG lane en masse or turned in one direction or the other and grew along the border of the lane. Importantly, support cells also moving from the DRGs did not correlate in any way with the front of growing or inhibited axons. Some DRGs had zero or only a few support cells, some had many and some had only support cell outgrowth. In each case of neurite contact with the lanes of KS/CS-PG, with or without support cells, there was complete neurite inhibition at 1 mg/ml of the proteoglycan (Fig. 18) (see below for analysis of actual concentration of KS/CS-PG bound to the nitrocellulose). Simultaneous observation of the RITC-labelled KS/CS-PG stripes with fluorescence microscopy and the DRG neurites with phase microscopy allowed us to see that the neurites responded distinctly at the proteoglycan stripe border (Fig. 18). Interestingly, cell bodies of DRG neurons which migrated out of the ganglia tended to stop along the border of the PG lane as did many of the support cells.

A concentration gradient of KS/CS-PG, ranging from 0.2 mg/ml to 1.0 mg/ml was tested within single culture dishes. We observed that numerous neurites crossed the lane if the concentration was low but fewer and fewer neurites crossed at the PG concentration of 0.2 mg/ml (left

side of photo) to 0.4 mg/ml (right side of photo) (compare to Figure 18 which shows complete inhibition at 1.0 mg/ml). The pattern of crossing at the lower concentrations of the PG was intermittent with expanses of complete inhibition in between the crossing points as analyzed from top to bottom along the border of the strip.

7.2.4. MODIFICATIONS OF THE DORSAL ROOT GANGLION ASSAY

7.2.4.1. KS/CS-PG:LN MIXTURES

We wished to further investigate the role that LN was playing in these assay cultures. The KS/CS-PG was bound to the nitrocellulose in the dishes, based on positive results of a dot blot immunoassay for keratan sulfate, however, it was unknown whether all of the stripe region was covered with the proteoglycan, or whether there was space left for the laminin to bind (either to the nitrocellulose coating or perhaps even to the proteoglycan itself) when the dish was covered sequentially first with the PG then with LN.

To be certain that laminin was directly incorporated into the stripe, we mixed laminin with KS/CS-PG before blotting onto the nitrocellulose. Immunocytochemistry using a polyclonal anti-laminin antibody showed that laminin was indeed present within the stripe. Additionally, we determined the maximum concentration of LN in combination with the proteoglycan which would still elicit the inhibitory effect. We learned that a ratio of 25 μg/ml laminin (or less) to 1 mg/ml KS/CS-PG was able to completely inhibit outgrowth (Fig. 20A), whereas, 50 or 100 μg/ml LN allowed increasing numbers of neurites to cross the KS/CS-PG + LN stripe (Fig. 20B). As a control, we tested the outgrowth abilities of the lowest test concentration of LN, in this case 10 μg/ml,

and observed that neurite outgrowth was extensive on this amount of LN when the KS/CS-PG was not present (Fig. 17B).

7.2.4.2. ACTUAL KS/CS-PG CONCENTRATION

Although it was known that the KS/CS-PG was adhered to the nitrocellulose from the dot blot immunoassay (data not shown), as well as from the behavior of the DRG neurites in the test dishes and controls, we were not certain as to the amount of proteoglycan that remained adsorbed to the nitrocellulose-coated petri dish during our inhibition assay. To determine the concentration of KS/CS-PG in the stripe, we prelabelled the sulfur of the proteoglycan with the radioisotope 35S. By comparing the counts received from our culture plates with that of pre-calculated standards of nitrocellulose alone and with buffer only, we determined that there was 0.25-1.0 μg/ml KS/CS-PG in the stripes following transferrance of the proteoglycan at 1 mg/ml in solution to the dish, including washes with media. We repeated this test with KS/CS-PG mixed with increasing concentrations of LN (KS/CS-PG + LN mixtures discussed below) from 0-100 μg/ml, to determine whether and at what rate the concentration of the proteoglycan might decrease as the LN concentration increases. We found somewhat unexpectedly that the concentration of the proteoglycan remained essentially unchanged regardless of the concentration of LN used. The binding characteristics of LN and KS/CS-PG to nitrocellulose may be complex and as yet undetermined interactions between the KS/CS-PG molecules and the LN molecules and the nitrocellulose may be occurring. Perhaps with increasing concentrations, the LN molecules may be packing more and more densely around the KS/CS-PG molecules. Also, since the LN in solution is present in such a low concentration in comparison to the PG, an effect on the PG concentration may not be observable until the LN

concentration is considerably increased. Whatever the reason, the result is fortunate, in that, we are able to evaluate neurite responses to a constant amount of KS/CS-PG while varying the concentration of LN in our assay.

7.2.4.3. KS/CS-PG:NCAM MIXTURES

In the above assay, LN was used as a stimulatory molecule for adhesion and elongation. However, based on immunostaining by us and others, we believe that, although LN in its extracellular form is present adjacent to the roof plate in the lateral walls of the spinal cord, it may be present only in very low concentrations or only in the cytoplasmic form within the roof plate itself. Therefore, we tested a molecular combination in the stripe assay using KS/CS-PG + polysialylated NCAM, shown previously to be expressed by the roof plate cells (Section 6, supra). The in vitro combination of KS/CS-PG + NCAM lanes alternating with LN lanes approximates the patterning of these particular molecules found in vivo (but possibly not the CS). We mixed 10 or 100 μg/ml polysialylated NCAM and 1 mg/ml KS/CS-PG, blotted these strips to the nitrocellulose, dried and applied 100 μg/ml LN evenly over the dish. We observed that the KS/CS-PG was significantly inhibitory with the addition of 10 μg/ml NCAM (FIG. 21) and that as much as 100 μg/ml NCAM still allowed essentially no crossing of neurites. Controls of 10-100 μg/ml NCAM alone (i.e. no KS/CS-PG) in the stripes alternated with LN demonstrated that this concentration of NCAM alone is a conducive substrate for DRG neurite outgrowth (Fig. 22. Analysis of the neurite pattern within and between the lanes did not reveal any differences between outgrowth on this form and NCAM versus outgrowth on LN. Interestingly, we did not find any differences in the neurite patterning at the interface where axons elongated from LN to NCAM or vice versa.

7.2.4.4. ENZYME DIGESTION ASSAYS

We analyzed the effect of individual components of the KS/CS-PG molecule. To do this, we digested various portions of the KS/CS-PG molecule using specific enzymes. An immediate problem was that the standard enzymes for this purpose are insufficient to completely degrade keratan sulfate or chondroitin sulfate chains from the protein core of bovine KS/CS-PG. In chick, however, a substantial digestion can be obtained. We therefore used chick KS/CS-PG, isolated from day 8 chick limb bud cartilage cultures for our digestion assays.

Chick KS/CS-PG was treated with either keratanase, chondroitin ABC lyase or both in solution, prior to blotting onto the dishes. Once digested, these reagents were mixed with 5-10 μg/ml LN. Cultures using these four reagents showed that (1) the undigested chick KS/CS-PG significantly inhibits neurite outgrowth (Fig. 23A) when used at the same concentration as bovine KS/CS-PG, (2) CS as well as KS chains are necessary for complete inhibition since some crossing occurs with the use of keratanase (Fig. 23B) or chondroitin ABC lyase alone (Fig. 24), and (3) the protein core of this proteoglycan molecule has no inhibitory effect on chick DRG neurites which is demonstrated by the use of both keratanase and chondroitin ABC lyase together (Fig. 24B). The fact that the protein core assay did not result in neurite inhibition argues that a mass action effect is not a significant factor which governs neurite behavior in this protocol.

In a second type of digestion assay, we compared the behavior of the DRG neurites in a dish in which the stripes contained an undigested KS/CS-PG + LN mixture with one which had keratanase or chondroitinase treatment, just prior to seeding the DRGs. Thus, these digestions were done in the culture dish, rather than in solution prior to blotting. This permutation confirmed the above findings

that a significant amount of outgrowth occurs across the lanes when keratanase or chondroitinase treatment preceded DRG seeding in comparison to controls. This also indicated that laminin was indeed present on the dish, and in concentration high enough to promote axon outgrowth when uninhibited.

7.2.4.5. CHONDROSARCOMA TUMOR CELL LINE CARTILAGE CHONDROITIN SULFATE PROTEOGLYCAN (CS-PG)

We tested a rat chondrosarcoma tumor cell line cartilage proteoglycan (RCS) (a type of CS-PG) mixed with 10 μg/ml LN, which is structurally like the bovine KS/CS-PG except for two alterations: (1) it lacks the KS chain region (Hascall, V. C., 1981, in Biology of Carbohydrates, Vol. 1, V. Ginsburg, ed., John Wiley & Sons, Inc., pp. 1-49) and (2) the CS chains exist in the C-4-S form (the bovine and chick KS/CS-PG's discussed above are C-6-S). Many of the DRG neurites observed in this modification stopped at the CS-PG border, but a considerable portion of the neurites often crossed the strip at a concentration of 1 mg/ml (Fig. 25) which, importantly, was sufficient for virtually complete inhibition with the bovine and chick KS/CS-PG. Thus, this CS-PG molecule is less effective than the bovine or chick KS/CS-PG in achieving DRG neurite inhibition. Since partial inhibition was observed, the data indicated that not only may the KS chains play a role in complete inhibition, but that CS can also be a major contributor to the repulsion of neurites. Consideration must be given here to the fact that neurite outgrowth in response to C-4-S in the RCS and C-6-S of the bovine and chick KS/CS-PG cannot be directly compared.

7.2.4.6. THE ROLE OF OTHER GLYCOSAMINOGLYCAN-CONTAINING PROTEOGLYCANS ON NEURITE INHIBITION

To test whether the inhibitory effects observed were specific to the glycosaminoglycans keratan sulfate and chondroitin sulfate, we used a bovine glycosaminoglycan, dermatan sulfate, in the proteoglycan form (DS-PG) as one choice of many other possible glycosaminoglycans. We found that this proteoglycan was much less inhibitory to DRG neurites than that observed for bovine or chick KS/CS-PG at the same concentration of 1 mg/ml, and was much like the response to the rat chondrosarcoma cartilage proteoglycan (RCS) shown in Figure 25.

7.3. DISCUSSION

We have shown that dorsal root ganglia neurites are inhibited in a concentration-dependent manner by keratan sulfate/chondroitin sulfate proteoglycan (KS/CS-PG) mixed with either laminin (LN) or neural cell adhesion molecule (NCAM), even though the LN or NCAM are present in concentrations which, by themselves, normally allow abundant neurite growth. Removal of either KS or CS by glycosaminoglycan-specific enzyme digestions allowed neurites to cross into the PG lanes to various degrees. When both glycosaminoglycans were removed from the proteoglycan, leaving only the protein core mixed with LN, neurite growth across the lanes was totally unimpeded, as occurs in controls containing only LN or NCAM. These results suggest that the KS and CS chains possess a neurite inhibitory character but the protein core does not. Use of the protein core alone also serves as a suitable control to argue that neurite inhibition is not simply due to a mass action effect.

7.3.1. GLIAL CELLS ARE CAPABLE OF EXPRESSING OR SEQUESTERING AXON ATTRACTIVE OR REPULSIVE MOLECULES SIMULTANEOUSLY

Our previous data (Section 6) showed that the glial cells of the roof plate express adhesive molecules such as SSEA-1, L2 (HNK-1) and NCAM on their surface. Since axons do not travel through the roof plate, these molecules may function to hold the glial cells together or to help tether them to their surrounding attachment points at the pial surface or the dorsal portion of the ventricle. The roof plate also expresses KS which we have shown in this in vitro study to be inhibitory to DRG neurites. Thus the glial cells of the roof plate in vivo are capable of simultaneously producing or sequestering molecules for cell-cell attachment as well as for cell repulsion. This scenario may also occur at other axon refractory sites in the CNS such as the chick sub-plate where large extracellular spaces bordered by glial cell processes and filled with CS proteoglycan-containing extracellular matrices have been described (Palmert et al., 1986, Society for Neurosci. Abst. 12:1334).

7.3.2. ALL GLYCOSAMINOGLYCANS ARE NOT FUNCTIONALLY EQUIVALENT WITH RESPECT TO NEURITE ELONGATION

Our results show that inhibitory differences exist between proteoglycans within the same species since bovine dermatan sulfate proteoglycan (DS-PG) produces a reduced amount of neurite inhibition in comparison to bovine KS/CS-PG at the same concentration. Although proteoglycans affect axons in a variable manner, taken together, our present studies using keratan sulfate-chondroitin sulfate-, and dermatan sulfate proteoglycan, combined with the results of other laboratories using other glycosaminoglycans, show that these molecules, in general, are inhibitory to neurite outgrowth and cell attachment.

A character of the glycosaminoglycan portion of the proteoglycans that could be responsible, in part, for the difference in their biological effect is the variability in the level and pattern of sulfation. The RCS proteoglycan consists of chondroitin sulfate in the form of C-4-S, as does the DS-PG (the iduronic acid of the DS chains can epimerize to glucuronic acid to make C-4-S chains and the DS-PG often consists of a large number of these chains (Hascall, V. C., 1981, Biology of Carbohydrates, Vol. 1:1-49; Heinegaard and Paulsson, 1984, Extracellular Matrix Biochemistry, 277-322)). The CS chains of bovine and chick KS/CS-PG, however, are in the form of C-6-S. Since the RCS proteoglycan and DS-PG produced similar results with respect to the degree of neurite inhibition and this degree of inhibition differed from the chick and bovine KS/CS-PG, it is feasible that this slight configurational change can be detected by growth cones, causing them to be inhibited to different extents.

7.3.3. ALL GLIAL CHANNELS ARE NOT ALIKE

The literature shows that in numerous developing axon systems in vertebrates, large matrix-filled extracellular channels surrounded by glial cell processes, like those of the roof plate and chick subplate boundary, usually foreshadow the route of the pioneering axons whose growth cones closely associate with the glial cell membrane (His, W., 1887, Arch. Anat. Physiol. Leipzig Anat. Abt. 92:368-378; Silver, J. and Robb, R. M., 1979, Dev. Biol. 68:175-190; Krayanek, S., 1980, The Anatomical Record 197:95-109; Silver, J., and Sidman, R. L., 1980, J. Comp. Neurol. 189:101-111; Nordlander, R., and Singer, M., 1982, Exp. Neurol. 75:221-228; Nakanishi, S., 1983, Dev. Biol. 95:305-316; Simpson, S., 1983, in Spinal Cord Reconstruction, C. C. Kao et al., eds., Raven Press, New

York, N.Y., pp. 151-162; Bork et al., 1987, J. Comp. Neurol. 264:147-158). Wide bored channels are likely to be formed by the hydrated glycosaminoglycans bound to the proteoglycans of the matrix (Margolis, et al., 1986, Ann. N.Y. Acad. Sci. 481:46-54; Rutka, et al., 1988, J. Neurosurg. 69:155-170). It is intriguing that this same type of preformed glial channel for axons is present in invertebrates as well (Jacobs, J. R., and Goodman, C. S., 1989, J. Neurosci. 9(7):2402-2411), suggesting that the basic molecular mechanism for building axon "highways" has been widely conserved during evolution. Clearly, however, the large extracellular spaces made by the glial cell processes of the rodent roof plate and chick subplate do not facilitate axonal elongation. Anatomically, the glial cells of axon pathways and barriers and their associated channels look alike, but functionally, they are clearly quite different. Why do axons grow along the glial walls of some channels and not others? Why are glycosaminoglycans used at all to create spaces in regions where axons grow if glycosaminoglycans are innately inhibitory to axon outgrowth? It seems that the answer lies not in the physical presence of the extracellular space itself, but in the molecular nature of the surface of the cells which border the channel or the extracellular matrix within the channel.

7.3.4. PROTEOGLYCANS CAN BE EITHER INHIBITORY OR MAY BY MODIFIED TO BECOME PERMISSIVE

Although we have shown that glycosaminoglycans produce varying amounts of inhibition, dependent upon concentration and type, we have also learned that glycosaminoglycans can be made to be growth permissive, i.e., the inhibitory effect of the proteoglycan can be reduced or completely masked if accompanied by an appropriate concentration of the growth-promoting molecule, laminin. NCAM was far less effective in counteracting the

proteoglycan-mediated inhibition. These results suggest that a growth cone can sample chemical differences in its environment and make motile "decisions" based on summation of its sampling (see Letourneau, P. C., 1975, Dev. Biol. 44:92-101). It appears then, that growth-promoting molecules can modify the effect of those molecules which normally function to inhibit neurite outgrowth and vice versa. Therefore, by varying the ratio of attractive or adhesive molecules to inhibitory molecules on or around glia or modifying their temporal appearance, a wide range of neurite patterns can be elicited. The range extends from complete separation between the glial border and all adjacent axons (e.g. the roof plate, chick subplate, and dorsal optic stalk), to partial separation (a pattern of intermittent crossing like that of the right lane of Figure 19, and which occurs in vivo in the commissural and spinothalamic axons of the floor plate, or thalamic afferents to the barrel fields of somatosensory cortex) to totally undeflected/rectilinear patterns of axons (like that of the proximal optic nerve).

8. DERMATAN SULFATE AND KERATAN SULFATE/ CHONDROITIN SULFATE PROTEOGLYCAN INHIBIT NEURITE OUTGROWTH OF A NEURON-LIKE CELL LINE IN VITRO

Dermatan sulfate proteoglycan (DS-PG) and keratan sulfate/chondroitin sulfate proteoglycan (KS/CS-PG) were found to inhibit neurite outgrowth from cells of the neuronal-like cell line, PC-12. The inhibition of neurite outgrowth was demonstrated at a concentration of 0.1 mg/ml (1.25 μM) DS-PG and 0.25 mg/ml (0.31 μM) KS/CS-PG.

8.1. MATERIALS AND METHODS

8.1.1. SUBSTRATE PREPARATION

Tissue culture Petri dishes (60 mm) were coated with nitrocellulose as described in Section 7.1.1., supra. Cellulose filter paper (Whatman #1) was cut into 350 μm

strips and used to blot various proteoglycans onto the nitrocellulose substrate. The strips were soaked in 20 µl of the desired proteoglycan mixture. A solution of 1 mg/ml laminin (LN) was then spread evenly across the dish with a bent glass Pasteur pipet. Quantitation of these procedures, and suitable controls, are described in detail in Section 7., supra.

Stripes were made on the nitrocellulose-coated culture dishes with mixtures of LN (40 µg/ml), and KS/CS-PG, or DS-PG at various concentrations.

8.1.2. PC-12 NEURON-LIKE CELL LINE PREPARATIONS

The PC-12 cells used for the experiment were grown in media composed of DMEM plus 10% Horse Serum, 5% Fetal Calf Serum and 30 µg/ml gentamycin, final concentration. Confluent plates were disaggregated with 0.25% trypsin.

8.1.3. ASSAY FOR INHIBITION OF NEURITE OUTGROWTH

Plates used for experimental procedures were seeded at approximately one-million cells per 60 mm plate. Media used in the experiments was supplemented with Nerve Growth Factor (NGF) at a final concentration of 50 ng/ml.

Stripes coated with protcogylcan which were completely inhibitory to neurite outgrowth were evaluated as (-), those allowing slight outgrowth (+/-), and those permissive to neurite outgrowth as (+).

8.2. RESULTS: EFFECT OF KS/CS-PG ON PC-12 CELL NEURITE OUTGROWTH

PC-12 cells were plated on a substratum containing different concentrations of proteoglycans and grown in the presence of NGF. Neurite outgrowth was evaluated 24, 48 and 96 hours later, and is reported in Table 1.

TABLE 1.
INHIBITION OF PC-12 OUTGROWTH
BY KS/CS-PG AND DS-PG

KS/CS-PG Concentration	24h	48h	96h
2.7 mg/ml (3.37 μM)	-	-	-
1.0 mg/ml (1.25 μM)	+/-	-	-
0.5 mg/ml (0.62 μM)	+/-	-	-
0.25 mg/ml (0.31 μM)	+/-	+/-	+/-
DS-PG Concentration	**24h**	**48h**	**96h**
0.8 mg/ml (10 μM)	-	-	-
0.4 mg/ml (5 μM)	+/-	-	-
0.2 mg/ml (2.5 μM)	+/-	+/-	-
0.1 mg/ml (1.25 μM)	+	+/-	+/-
0.05 mg/ml (0.62 μM)	+	+	+

8.3. DISCUSSION

These data suggest that both DS and KS/CS-PGs are inhibitory to neurite outgrowth of PC-12 cells. PC-12 cells displayed a greater sensitivity to KS/CS-PG than to DS-PG on a molar basis, although on a per weight basis, the different protoglycan compositions show comparable inhibition. This latter observation corresponds with the comparative inhibitory effects of KS/CS-PG and DS-PG on neurite outgrowth of dorsal root ganglia neurons. DS-PG appeared to be less inhibitory on a molar basis than KS/CS-PG for DRG neurons (Section 7.2.4.6., supra, Section 9.2, infra). Thus, the growth cones of PC-12 neuron-like cells appear to share the same specificity for proteoglycan inhibition that DRG neurons demonstrate. As discussed in

Section 7.3.2., supra, growth cones from both cell types may demonstrate a configurational specificity for C-4-S over C-6-S.

9. EFFECT OF DS-PG ON DRG NEURITE OUTGROWTH

KS/CS-PG was shown to inhibit neurite outgrowth of dorsal root ganglia (DRG from chick E6, Section 7.2.1., supra). In this example DS-PG inhibition of neurite outgrowth was assayed.

9.1. MATERIALS AND METHODS

DRGs were prepared as described in Section 7.1.2., supra. DS-PG stripes were prepared on laminim-coated nitrocellulose as described in Sections 7.1.1. and 8.1.1., supra.

The assay for the inhibition of outgrowth was performed as described in Section 8.1.3., supra. DRGs (Chick E 6 dorsal root ganglia) were plated on a substratum containing different concentrations of dermatan sulfate proteoglycan on 100 μg/ml laminin and grown in the presence of Nerve Growth Factor (NGF).

Stripes coated with DS-PG which were completely inhibitory to neurite outgrowth were evaluated as (-), those allowing slight outgrowth (+/-), and those permissive to neurite outgrowth as (+).

9.2. RESULTS

E-6 dorsal root ganglia (DRG) cells were cultured on nitrocellulose treated with a dermatan sulfate proteoglycan (DS-PG) strip. The results of the growth assay are shown in Table 2.

TABLE 2.
INHIBITION OF DRG OUTGROWTH
AFTER 24 HOURS ON DS-PG

DS-PG Concentration and Assay Conditions	Neurite Outgrowth
0.1 mg/ml DS-PG	+
0.2 mg/ml DS-PG	+/-
0.4 mg/ml DS-PG	-
0.8 mg/ml DS-PG	-

DRG neurite outgrowth was completely inhibited by as little as 0.4 mg/ml (5 μM) DS-PG, and partly inhibited by 0.2 mg/ml (2.5 μM) DS-PG.

9.3. DISCUSSION

These results suggest that DRG cells are slightly more sensitive to inhibition by DS-PG than the neuron-like cell line PC-12. DRG cells are partly inhibited from outgrowth by as little as 0.2 mg/ml DS-PG, whereas PC-12 cells were partly inhibited by 0.4 mg/ml DS-PG.

On a molar basis, the DRG cells are more sensitive to KS/CS-PG than to DS-PG, as reported in Section 7.2.4.6., supra. These results correspond to the results with the PC-12 cell line, which was also more sensitive to KS/CS-PG than to DS-PG on a molar basis.

10. DERMATAN SULFATE AND KERATAN SULFATE/ CHONDROITIN SULFATE INHIBIT GLIAL CELL INVASION IN VITRO

Dermatan sulfate proteoglycan (DS-PG) and keratan sulfate/chondroitin sulfate proteoglycan (KS/CS-PG) were found to inhibit glial cell and astrocyte invasion. C-6 rat glial tumor cells and MCG-28 young immortalized mouse astrocytes were unable to invade the proteoglycan coated substratum for up to 96 hours.

10.1. MATERIALS AND METHODS

10.1.1. SUBSTRATE PREPARATION

Tissue culture Petri dishes (60-mm) were coated with nitrocellulose (Schleicher & Schuell, Type BA 85 : 0.5 ml of a 5 cm^2 section dissolved in 6 ml methanol). Cellulose filter paper (Whatman #1) was cut into 350 μm strips and used to blot various proteoglycans onto the nitrocellulose substrate. The strips were soaked in 20 μl of the desired proteoglycan mixture. A solution of 1 mg/ml laminin (LN) was then spread evenly across the dish with a bent glass Pasteur pipet. These methods are also described in Section 7.1.1, supra.

Stripes were made on the nitrocellulose-coated culture dishes with mixtures of LN (40 μg/ml), and KS/CS-PG or DS-PG at various concentrations.

10.1.2. PREPARATION OF CELL LINES

The different cell lines used for the experiment were grown in media composed of DMEM plus 5% Fetal Bovine Serum, 5% Calf Serum and 30 μg/ml gentamycin. Confluent plates were disaggregated with 0.25% trypsin. Plates used for experimental procedures were seeded at a ratio of 1:6 from confluent plates. C-6 rat glial tumor cells (Paganetti et al, 1988, J. Cell Biol. 107:2291-2291) and

MCG-28 young immortalized mouse astrocytes (a murine neonatal astrocyte line immortalized with SV-40), were utilized for these experiments.

10.1.3. ASSAY FOR INHIBITION OF OUTGROWTH

Stripes coated with proteoglycan which were completely inhibitory to cell invasion were evaluated as (-), those allowing slight invasion (+/-), and those permissive to invasion as (+) (Figure 26).

10.2. RESULTS

10.2.1. EFFECT OF DS-PG ON C-6 AND MCG-28 CELL MIGRATION AND INVASION

C-6 glial cells were plated on different concentrations of DS-PG. Plates were evaluated after 3, 24 and 48 hours, and 5 days. The results are shown in Table 3.

TABLE 3.

INHIBITION OF C-6 CELL INVASION ON DS-PG

DS-PG Concentration	3 hrs	24 hrs	48 hrs	5 days
0.8 mg/ml (10 μM)	-	-	-	-
0.4 mg/ml (5 μM)	-	-	-	-
0.2 mg/ml (2.5 μM)	+/-	+	+	+
0.1 mg/ml (1.25 μM)	+	+	+	+

MCG-28 immortalized young astrocytes were plated on different concentrations of DS-PG. Plates were evaluated 24, 48, and 72 hours, and 5 days later. The results are shown in Table 4.

TABLE 4.

INHIBITION OF MCG-28 CELL INVASION ON DS-PG

DS-PG Concentration	24 hrs	48 hrs	72 hrs	5 days
0.8 mg/ml (10 μM)	-	-	-	-
0.4 mg/ml (5 μM)	-	-	-	+/-
0.2 mg/ml (2.5 μM)	+/-	+	+	+
0.1 mg/ml (1.25 μM)	+	+	+	+

At the concentration of between 0.4 and 0.2 mg/ml DS-PG, the cell lines are no longer inhibited and can invade the introcellulose strip. DS-PG inhibited outgrowth of both cell lines comparably; no obvious enhancement of inhibition of glial cells (C-6) or astrocytes (MCG-28) was observed.

10.2.2. EFFECT OF KS/CS-PG ON C-6 AND MCG 28 CELL MIGRATION AND INVASION

C-6 and MCG-28 cells were grown on various concentrations of KS/CS-PG, and evaluated at several time points later. The results are shown in Table 5.

TABLE 5.
INHIBITION OF C-6 AND MCG-28
CELL INVASION BY KS/CS-PG

a. C-6	KS/CS-PG Concentration	24 hrs	48 hrs	5 days
	2.7 mg/ml (3.37 μM)	+/-	+/-	+
	1.0 mg/ml (1.25 μM)	+	+	+
	0.5 mg/ml (0.62 μM)	+	+	+
b. MCG-28	KS/CS-PG Concentration	24 hrs	48 hrs	5 days
	2.7 mg/ml (3.37 μM)	+/-	+/-	+
	1.0 mg/ml (1.25 μM)	+/-	+	+
	0.5 mg/ml (0.62 μM)	+/-	+	+
	0.25 mg/ml (0.31 μM)	+	+	+

10.2.3. COMPARISON OF KS/CS-PG AND DS-PG ON CELL MIGRATION AND INVASION

The different cell lines were grown on either KS/CS-PG or DS-PG coated stripes. Plates were evaluated for the extent of cell invasion after 24 and 48 hours in culture. The results for growth on KS/CS-PG are shown in Table 6. The results for growth on DS-PG are shown in Table 7.

TABLE 6.
COMPARISON OF INHIBITION OF C-6
AND MCG-28 CELL INVASION BY KS/CS-PG

Cell Line	KS/CS-PG 1.0 mg/ml (1.25 μM)	
	24 hrs	48 hrs
C-6	+	+
MCG-28	+/-	+

TABLE 7.
COMPARISON OF INHIBITION OF C-6
AND MCG-28 CELL INVASION BY DS-PG

Cell Line	0.8 mg/ml (10 μM)		0.1 mg/ml (1.25 μM)	
	24 hrs	48 hrs	24 hrs	48 hrs
C-6	-	-	+	+
MCG-28	-	-	+	+

10.3. DISCUSSION

DS-PG is a more potent inhibitor than KS/CS-PG when compared at equal dry weight concentration. C-6 and MCG-28 cells grown on 1.0 mg/ml (1.25 μM) KS/CS-PG exhibited invasion by 24 hours (Table 6), whereas inhibition of invasion on 0.8 mg/ml (10 μM) of DS-PG was maintained for at least 4 days (Tables 3 and 4). At equal molar concentrations the KS/CS- and DS-PGs were found to inhibit outgrowth comparably. This result contrasts with the observations for dorsal root ganglia neurons (Section

9.2., supra) and the neuron-like cell line PC-12 (Section 8.2., supra), in which KS/CS-PG acted as a more potent inhibitor on a molar basis than DS-PG. On a dry weight basis, KS/CS-PG and DS-PG show comparable inhibition of neurite outgrowth.

These results indicate that glial cells, including astrocytes, have a slightly different specificity for proteoglycan inhibition than neurons. Presumably, neurite outgrowth and glial cell migration or invasion will be inhibited to different degrees, possibly depending on the composition of proteoglycan in the local matrix. Thus, proteoglycans may exert a fine regulatory action on the growth of neurons and non-neuronal cells in vivo, thus spacially regulating cell growth.

Various references are cited herein, the disclosures of which are incorporated by reference herein in their entireties.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising an effective amount of a molecule, which molecule comprises keratan sulfate disaccharide; and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1 in which the molecule comprises keratan sulfate proteoglycan.

3. The pharmaceutical composition of claim 1 in which the molecule comprises keratan sulfate glycosaminoglycan.

4. A pharmaceutical composition comprising an effective amount of a molecule, which molecule comprises chondroitin sulfate disaccharide; and a pharmaceutically acceptable carrier.

5. The pharmaceutical composition of claim 4 in which the molecule comprises chondroitin sulfate proteoglycan.

6. The pharmaceutical composition of claim 4 in which the molecule comprises chondroitin sulfate glycosaminoglycan.

7. A pharmaceutical composition comprising an effective amount of a molecule, which molecule comprises dermatan sulfate disaccharide; and a pharmaceutically acceptable carrier.

8. The pharmaceutical composition of claim 7 in which the molecule comprises dermatan sulfate proteoglycan.

9. The pharmaceutical composition of claim 7 in which the molecule comprises dermatan sulfate glycosaminoglycan.

10. The pharmaceutical composition of claim 7 in which the dermatan sulfate has a C-4 sulfer linkage.

11. The pharmaceutical composition of claim 1, 2 or 3 which further comprises a second molecule comprising a compound selected from the group consisting of chondroitin sulfate disaccharide, chondroitin sulfate glycosaminoglycan, and chondroitin sulfate proteoglycan.

12. The pharmaceutical composition of claim 1, 2 or 3 which further comprises a second molecule comprising a compound selected from the group consisting of dermatan sulfate disaccharide, dermatan sulfate glycosaminoglycan, and dermatan sulfate proteoglycan.

13. The pharmaceutical composition of claim 4 in which the chondroitin sulfate has a C-6 sulfur linkage.

14. The pharmaceutical composition of claim 1, 2 or 3 in which the keratan sulfate is Type I (corneal).

15. The pharmaceutical composition of claim 1, 2 or 3 in which the keratan sulfate is Type II (skeletal).

16. A pharmaceutical composition comprising an effective amount of a molecule which antagonizes or destroys the growth inhibitory function of keratin sulfate disaccharide, keratan sulfate glycosaminoglycan or keratan sulfate proteoglycan; and a pharmaceutically acceptable carrier.

17. A pharmaceutical composition comprising an effective amount of a molecule which antagonizes or destroys the growth inhibitory function of chondroitin sulfate disaccharide, chondroitin sulfate glycosaminoglycan or chondroitin sulfate proteoglycan; and a pharmaceutically acceptable carrier.

18. A pharmaceutical composition comprising an effective amount of a molecule which antagonizes or destroys the growth inhibitory function of dermatan sulfate disaccharide, dermatan sulfate glycosaminoglycan or dermatan sulfate proteoglycan; and a pharmaceutically acceptable carrier.

19. A pharmaceutical composition comprising an effective amount of an antibody to keratan sulfate, or a fragment or derivative thereof containing the binding domain; and a pharmaceutically acceptable carrier.

20. A pharmaceutical composition comprising an effective amount of an antibody to chondroitin sulfate, or a fragment or derivative thereof containing the binding domain; and a pharmaceutically acceptable carrier.

21. A pharmaceutical composition comprising an effective amount of an antibody to dermatan sulfate, or a fragment or derivative thereof containing the binding domain; and a pharmaceutically acceptable carrier.

22. The pharmaceutical composition of claim 19 in which the antibody is a monoclonal antibody.

23. The pharmaceutical composition of claim 20 in which the antibody is a monoclonal antibody.

24. The pharmaceutical composition of claim 21 in which the antibody is a monoclonal antibody.

25. The pharmaceutical composition of claim 22 in which the monoclonal antibody is selected from the group consisting of MZ15, 1/20/5-D-4, 4/8/1-B-4, 4-D-1, and 8-C-2.

26. A pharmaceutical composition comprising an effective amount of an enzyme which degrades keratan sulfate; and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising an effective amount of an enzyme which degrades chondroitin sulfate; and a pharmaceutically acceptable carrier.

28. A pharmaceutical composition comprising an effective amount of an enzyme which degrades dermatan sulfate; and a pharmaceutically acceptable carrier.

29. The pharmaceutical composition of claim 26 in which the enzyme is selected from the group consisting of endo-b-galactosidase and keratanase.

30. The pharmaceutical composition of claim 27 in which the enzyme is selected from the group consisting of chondroitinase and chondroitin ABC lyase.

31. The pharmaceutical composition of claim 28 in which the enzyme is selected from the group consisting of chondroitin ABC lyase.

32. The pharmaceutical composition of claim 26 which further comprises an effective amount of an enzyme which degrades chondroitin sulfate.

33. The pharmaceutical composition of claim 29 which further comprises an effective amount of an enzyme which degrades chondroitin sulfate.

34. The pharmaceutical composition of claim 26 which further comprises an effective amount of an enzyme which degrades dermatan sulfate.

35. The pharmaceutical composition of claim 29 which further comprises an effective amount of an enzyme which degrades dermatan sulfate.

36. The pharmaceutical composition of claim 33 which further comprises an effective amount of an enzyme which degrades dermatan sulfate.

37. A method for treatment of a patient in whom inhibition of nerve growth is desired comprising administering to the patient an effective amount of a molecule comprising keratan sulfate disaccharide.

38. A method for treatment of a patient in whom inhibition of glial cell migration or invasion is desired comprising administering to the patient an effective amount of a molecule comprising keratan sulfate disaccharide.

39. The method according to claim 37 in which the molecule comprises keratan sulfate proteoglycan.

40. The method according to claim 38 in which the molecule comprises keratan sulfate proteoglycan.

41. The method according to claim 37 in which the molecule comprises keratan sulfate glycosaminoglycan.

42. The method according to claim 38 in which the molecule comprises karatan sulfate glycosaminoglycan.

43. A method for treatment of a patient in whom inhibition of nerve growth is desired comprising administering to the patient an effective amount of a molecule comprising chondroitin sulfate disaccharide.

44. A method for treatment of a patient in whom inhibition of glial cell migration or invasion is desired comprising administering to the patient an effective amount of a molecule comprising chondroitin sulfate disaccharide.

45. The method according to claim 43 in which the molecule comprises chondroitin sulfate proteoglycan.

46. The method according to claim 44 in which the molecule comprises chondroitin sulfate proteoglycan.

47. The method according to claim 43 in which the molecule comprises chondroitin sulfate glycosaminoglycan.

48. The method according to claim 44 in which the molecule comprises chondroitin sulfate glycosaminoglycan.

49. The method according to claim 38, 40, 42, 44, 46, or 48 in which the glial cell is an astrocyte.

50. A method for treatment of a patient in whom inhibition of nerve growth is desired, comprising administering to the patient an effective amount of a molecule comprising dermatan sulfate disaccharide.

51. A method for treatment of a patient in whom inhibition of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of a molecule comprising dermatan sulfate disaccharide.

52. The method according to claim 50 in which the molecule comprises dermatan sulfate proteoglycan.

53. The method according to claim 51 in which the molecule comprises dermatan sulfate proteoglycan.

54. The method according to claim 50 in which the molecule comprises dermatan sulfate glycosaminoglycan.

55. The method according to claim 51 in which the molecule comprises dermatan sulfate glycosaminoglycan.

56. The method according to claim 51, 53, or 55 in which the glial cell is an astrocyte.

57. The method according to claim 37, 39, or 41 in which the patient has a glioma.

58. The method according to claim 37, 39, or 41 in which the patent has a tumor of nerve tissue.

59. The method according to claim 58 in which the tumor is a neuroblastoma.

60. The method according to claim 37, 39, or 41 in which the patient has a neuroma.

61. The method according to claim 37, 39, or 41 which further comprises administering to the patient an effective amount of a second molecule comprising a compound

selected from the group consisting of chondroitin sulfate disaccharide, chondroitin sulfate glycosaminoglycan, and chondroitin sulfate proteoglycan.

62. The method according to claim 37, 39, or 41 which further comprises administering to the patient an effective amount of a second molecule comprising a compound selected from the group consisting of dermatan sulfate disaccharide, dermatan sulfate glycosaminoglycan, and dermatan sulfate proteoglycan.

63. The method according to claim 61 in which the chondroitin sulfate has a C-6 sulfur linkage.

64. The method according to claim 37, 39, or 41 in which the keratan sulfate is Type I (corneal).

65. The method according to claim 37, 39, 41 in which the keratan sulfate is Type II (skeletal).

66. The method according to claim 50, 52, or 54 in which the patient has a glioma.

67. The method according to claim 50, 52, or 54 in which the patient has a tumor of nerve tissue.

68. The method according to claim 67 in which the tumor is a neuroblastoma.

69. The method according to claim 50, 52, or 54 in which the patient has a neuroma.

70. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an

effective amount of a molecule which antagonizes or destroys the nerve growth inhibitory function of keratan sulfate disaccharide, keratan sulfate glycosaminoglycan or keratan sulfate proteoglycan.

71. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroys the glial cell migration or invasion inhibitory function of keratan sulfate disaccharide, keratan sulfate glycosaminoglycan or keratan sulfate proteoglycan.

72. The method of claim 71 in which the glial cell is an astrocyte.

73. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroys the nerve growth inhibitory function of chondroitin sulfate disaccharide, chondroitin sulfate glycosaminoglycan or chondroitin sulfate proteoglycan.

74. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroys the glial cell migration or invasion inhibitory function of chondroitin sulfate disaccharide, chondroitin sulfate glycosaminoglycan or chondroitin sulfate proteoglycan.

75. The method of claim 74 in which the glial cell is an astrocyte.

76. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroyes the nerve growth inhibitory function of dermatan sulfate disaccharide, dermatan sulfate glycosaminoglycan or dermatan sulfate proteoglycan.

77. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroyes the glial cell migration or invasion inhibitory function of dermatan sulfate disaccharide, dermatan sulfate glycosaminoglycan or dermatan sulfate proteoglycan.

78. The method of claim 77 in which the glial cell is an astrocyte.

79. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of an antibody to keratan sulfate, or a fragment or derivative thereof containing the binding domain.

80. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering

to the patient an effective amount of an antibody to keratan sulfate, or a fragment or derivative thereof containing the binding domain.

81. The method of claim 80 in which the glial cell is an astrocyte.

82. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of an antibody to chondroitin sulfate, or a fragment or derivative thereof containing the binding domain.

83. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of an antibody to chondroitin sulfate, or a fragment or derivative thereof containing the binding domain.

84. The method of claim 83 in which the glial cell is an astrocyte.

85. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of an antibody to dermatan sulfate, or a fragment or derivative thereof containing the binding domain.

86. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering

to the patient an effective amount of an antibody to dermatan sulfate, or a fragment or derivative thereof containing the binding domain.

87. The method of claim 86 in which the glial cell is an astrocyte.

88. The method according to claim 79, 80, or 81 in which the antibody is a monoclonal antibody.

89. The method according to claim 88 in which the monoclonal antibody is selected from the group consisting of MZ15, 1/20/5-D-4, 4/8/1-B-4, 4-D-1, and 8-C-2.

90. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of an enzyme which degrades keratan sulfate.

91. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of an enzyme which degrades keratan sulfate.

92. The method of claim 91 in which the glial cell is an astrocyte.

93. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of an enzyme which degrades chondroitin sulfate.

94. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of an enzyme which degrades chondroitin sulfate.

95. The method of claim 94 in which the glial cell is an astrocyte.

96. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of an enzyme which degrades dermatan sulfate.

97. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of an enzyme which degrades dermatan sulfate.

98. The method according to claim 97 in which the glial cell is an astrocyte.

99. The method according to claim 90, 91, or 92 in which the enzyme is selected from the group consisting of endo-b-galactosidase and keratanase.

100. The method according to claim 93, 94, or 95 in which the enzyme is selected from the group consisting of chondroitinase and chondroitin ABC lyase.

101. The method according to claim 96, 97, or 98 in which the enzyme is selected from the group consisting of chondiotin ABC lyase.

102. The method according to claim 90, 91, or 92 which further comprises administering to the patient an effective amount of an enzyme which degrades chondroitin sulfate.

103. The method according to claim 99 which further comprises administering to the patient an effective amount of an enzyme which degrades chondroitin sulfate.

104. The method according to claim 90, 91, or 92 which further comprises administering to the patient an effective amount of an enzyme which degrades dermatan sulfate.

105. The method according to claim 99 which further comprises administering to the patient an effective amount of an enzyme which degrades dermatan sulfate.

106. The method according to claim 102 which further comprises administering to the patient an effective amount of an enzyme which degrades dermatan sulfate.

107. The method according to claim 70, 79, or 90 in which the nerve damage is caused by a disease or disorder selected from the group consisting of trauma, surgery, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, exposure to toxic agents and degenerative disorders of the nervous system.

108. The method according to claim 71, 80 or 91 in which the glial cell damage is caused by a disease or disorder selected from the group consisting of trauma, surgery, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, exposure to toxic agents and degenerative disorders.

109. The method of claim 108 in which the glial cell is an astrocyte.

110. The method according to claim 73, 82, 93 in which the nerve damage is caused by a disease or disorder selected from the group consisting of trauma, surgery, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, exposure to toxic agents and degenerative disorders of the nervous system.

111. The method according to claim 74, 83, or 94 in which the glial cell damage is caused by a disease or disorder selected from the group consisting of trauma, surgery, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, exposure to toxic agents and degenerative disorders.

112. The method of claim 111 in which the glial cell is an astrocyte.

113. The method according to claim 76, 85, or 96 in which the nerve damage is caused by a disease or disorder selected from the group consisting of trauma, surgery, ischemia, infection, metabolic disease, nutritional deficency, malignancy, exposure to toxic agents and degenerative disorders of the nervous system.

114. The method according to claim 77, 86, or 97 in which the glial cell damage is caused by a disease or disorder selected from the group consisting of trauma, surgery, ischemia, infection, metabolic disease, nutritional deficency, malignancy, exposure to toxic agents and degenerative disorders.

115. The method of claim 117 in which the glial cell is an astrocyte.

116. The method according to claim 70, 79, or 90 in which the nerve damage is caused by a degenerative disorder of the nervous system selected from the group consisting of Alzheimer's disease, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy, and peripheral neuropathies.

117. The method according to claim 73, 82, or 93 in which the nerve damage is caused by a degenerative disorder of the nervous system selected from the group consisting of Alzheimer's disease, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy, and peripheral neuropathies.

118. The method according to claim 76, 85, or 96 in which the nerve damage is caused by a degenerative disorder of the nervous system selected from the group consisting of Alzheimer's disease, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy, and peripheral neuropathies.

119. The method according to claim 38, 40, or 42 in which the glial cell migration or invasion is caused by a disease or disorder selected from the group consisting of trauma, surgery, viral infection, bacterial infection, metabolic disease, malignancy, exposure to toxic agents, and hyperplastic situations.

120. The method according to claim 44, 46, or 48 in which the glial cell migration or invasion is caused by a disease or disorder selected from the group consisting of trauma, surgery, viral infection, bacterial infection, metabolic disease, malignancy, exposure to toxic agents, and hyperplastic situations.

121. The method according to claim 51, 53, or 55 in which the glial cell migration or invasion is caused by a disease or disorder selected from the group consisting of trauma, surgery, viral infection, bacterial infection, metabolic disease, malignancy, exposure to toxic agents, and hyperplastic situations.

122. A method to protect an organ or tissue from glial cell invasion comprising coating the organ or tissue with an effective amount of a molecule selected from the group consisting of keratan sulfate disaccharide, keratan sulfate proteoglycan, and keratan sulfate glycosaminoglycan.

123. A method to protect an organ or tissue from glial cell invasion comprising coating the organ or tissue with an effective amount of a molecule selected from the group consisting of chondroitin sulfate disaccharide, chondroitin sulfate proteoglycan, and chondroitin sulfate glycosaminoglycan.

124. A method to specifically protect an organ or tissue from glial cell invasion comprising coating the organ or tissue with an effective amount of a molecule from the group consisting of dermatan sulfate disaccharide, dermatan sulfate proteoglycan, and dermatan sulfate glycosaminoglycan.

125. The method according to claim 122, 123, or 124 in which the organ or tissue is selected from the group consisting of dorsal root ganglia, optic nerve, and optic chiasma.

126. The method according to claim 122, 123, or 124 in which the glial cell is an astrocyte.

127. The method according to claim 125 in which the glial cell is an astrocyte.

128. A pharmaceutical composition comprising an effective amount of a molecule, which molecule comprises heparan sulfate disaccharide; and a pharmaceutically acceptable carrier.

129. The pharmaceutical composition of claim 128 in which the molecule comprises heparan sulfate proteoglycan.

130. The pharmaceutical composition of claim 128 in which the molecule comprises heparan sulfate glycosaminoglycan.

131. A pharmaceutical composition comprising an effective amount of a molecule, which molecule comprises heparin disaccharide; and a pharmaceutically acceptable carrier.

132. The pharmaceutical composition of claim 131 in which the molecule comprises heparin proteoglycan.

133. The pharmaceutical composition of claim 131 in which the molecule comprises heparin glycosaminoglycan.

134. A pharmaceutical composition comprising an effective amount of a molecule, which molecule comprises hyaluronate disaccharide; and a pharmaceutically acceptable carrier.

135. The pharmaceutical composition of claim 126 in which the molecule comprises hyaluronate glycosaminoglycan.

136. A pharmaceutical composition comprising an effective amount of a molelcule which antagonizes or destroys the growth inhibitory function of heparan sulfate disaccharide, heparan sulfate glycosaminoglycan, or heparan sulfate proteoglycan.

137. A pharmaceutical composition comprising an effective amount of a molelcule which antagonizes or destroys the growth inhibitory function of heparin disaccharide, heparin glycosaminoglycan, or heparin proteoglycan.

138. A pharmaceutical composition comprising an effective amount of a molelcule which antagonizes or destroys the growth inhibitory function of hyaluronate disaccharide, or hyaluronate glycosaminoglycan.

139. A method for treatment of a patient in whom inhibition of nerve growth is desired comprising administering to the patient an effective amount of a molecule from the group consisting of heparan sulfate disaccharide, heparan sulfate proteoglycan, or heparan sulfate glycosaminoglycan.

140. A method for treatment of a patient in whom inhibition of nerve growth is desired comprising administering to the patient an effective amount of a molecule from the group consisting of heparin disaccharide, heparin proteoglycan, or heparin glycosaminoglycan.

141. A method for treatment of a patient in whom inhibition of nerve growth is desired comprising administering to the patient an effective amount of a molecule from the group consisting of hyaluronate disaccharide, or hyaluronate glycosaminoglycan.

142. A method for treatment of a patient in whom inhibition of glial cell migration or invasion is desired comprising administering to the patient an effective amount of a molecule from the group consisting of heparan sulfate disaccharide, heparan sulfate proteoglycan, or heparan sulfate glycosaminoglycan.

143. A method for treatment of a patient in whom inhibition of glial cell migration or invasion is desired comprising administering to the patient an effective amount of a molecule from the group consisting of heparin disaccharide, heparin proteoglycan, or heparin glycosaminoglycan.

144. A method for treatment of a patient in whom inhibition of glial cell migration or invasion is desired comprising administering to the patient an effective amount of a molecule from the group consisting of hyaluronate disaccharide, or hyaluronate glycosaminoglycan.

145. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an

effective amount of a molecule which antagonizes or destroys the nerve growth inhibitory function of heparan sulfate disaccharide, heparan sulfate proteoglycan, or heparan sulfate glycosaminoglycan.

146. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroys the nerve growth inhibitory function of heparin disaccharide, heparin proteoglycan, or heparin glycosaminoglycan.

147. A method for treatment of a patient with nerve damage or in whom promotion of nerve growth is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroys the nerve growth inhibitory function of hyaluronate disaccharide, or hyaluronate glycosaminoglycan.

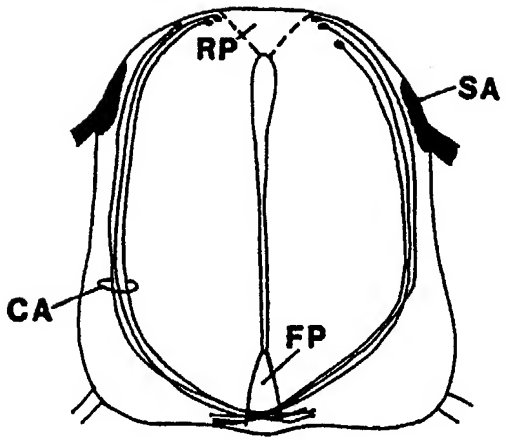
148. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroys the glial cell migration or invasion inhibitory function of heparan sulfate disaccharide, heparan sulfate proteoglycan, or heparan sulfate glycosaminoglycan.

149. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of a molecule which

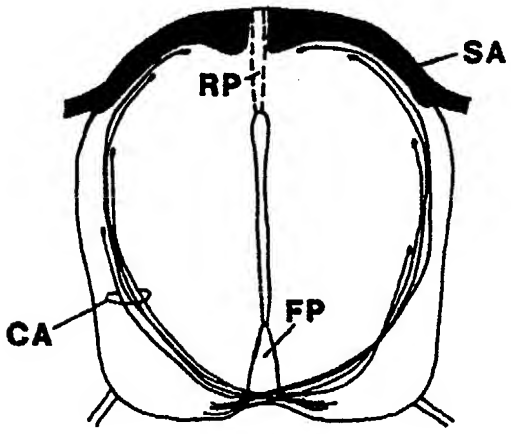
antagonizes or destroys the glial cell migration or invasion inhibitory function of heparin disaccharide, heparin proteoglycan, or heparin glycosaminoglycan.

150. A method for treatment of a patient with glial cell damage or in whom promotion of glial cell migration or invasion is desired, comprising administering to the patient an effective amount of a molecule which antagonizes or destroys the glial cell migration or invasion inhibitory function of hyaluronate disaccharide, or hyaluronate glycosaminoglycan.

FIG. 1



E13.5



E15.5

FIG. 2A

FIG. 2B

FIG. 2C

FIG. 3A

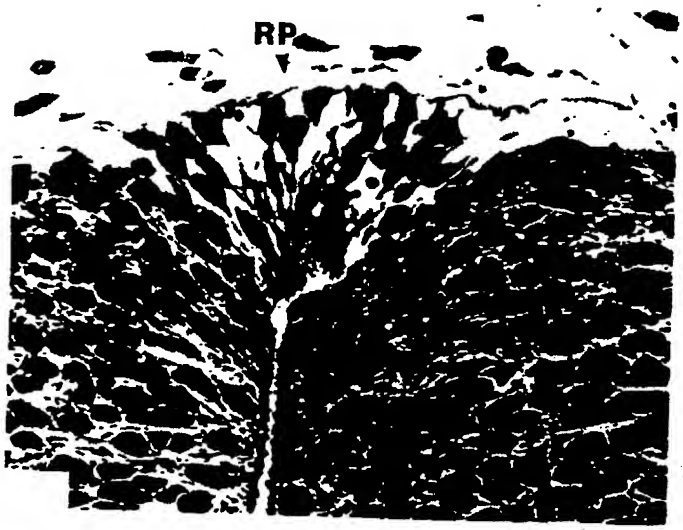


FIG. 3B

FIG. 4

FIG. 5

FIG. 6A

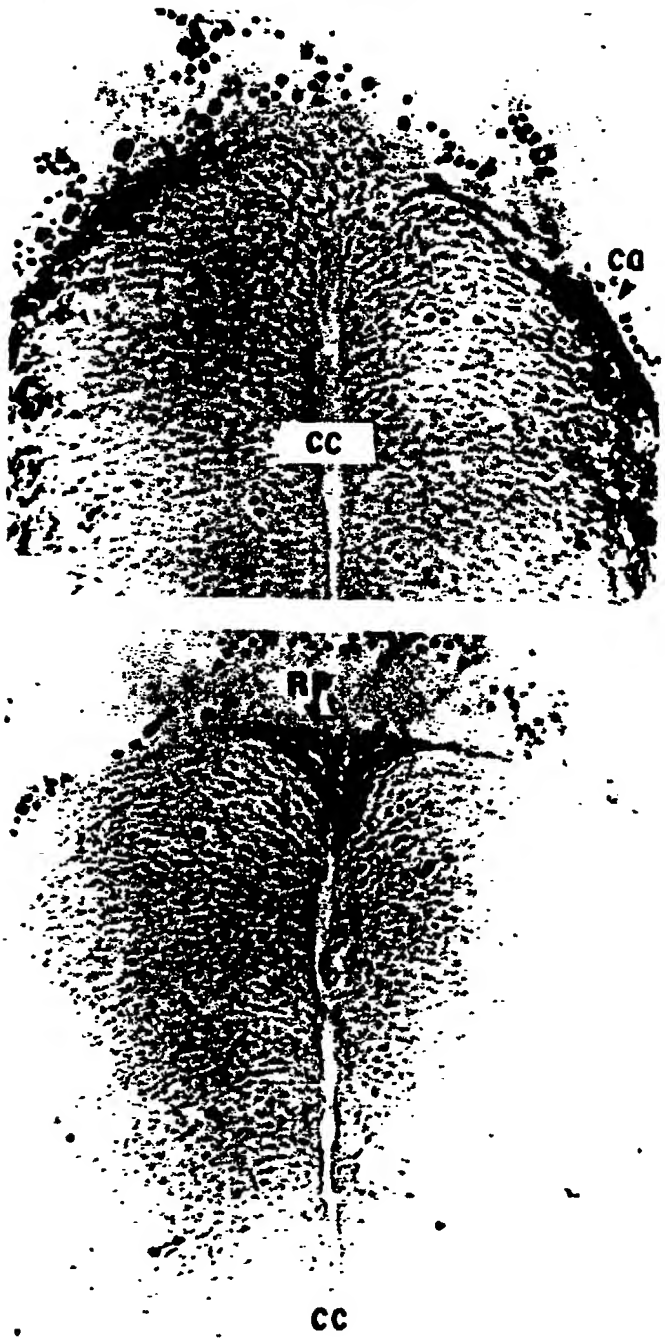


FIG. 6B

FIG.7C

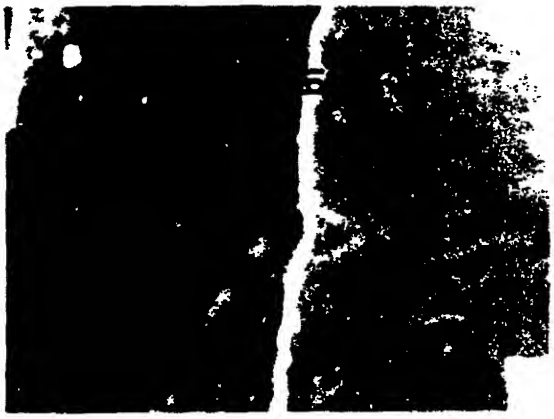


FIG.7B

FIG.7A

FIG. 8A

FIG. 8B

FIG. 9A

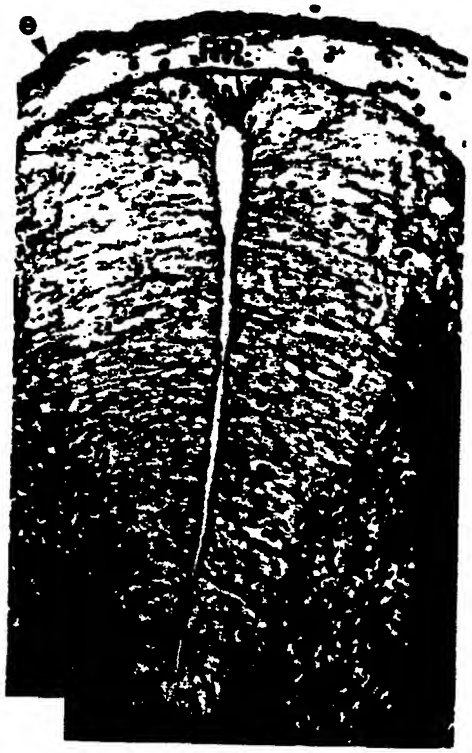


FIG. 9B

FIG. 9C

FIG. 9D

FIG.10C

FIG.10B

FIG.10A

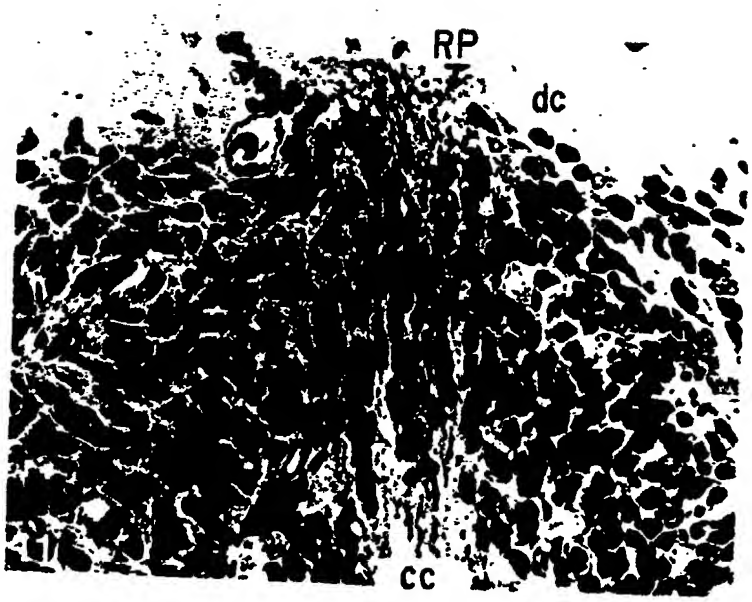
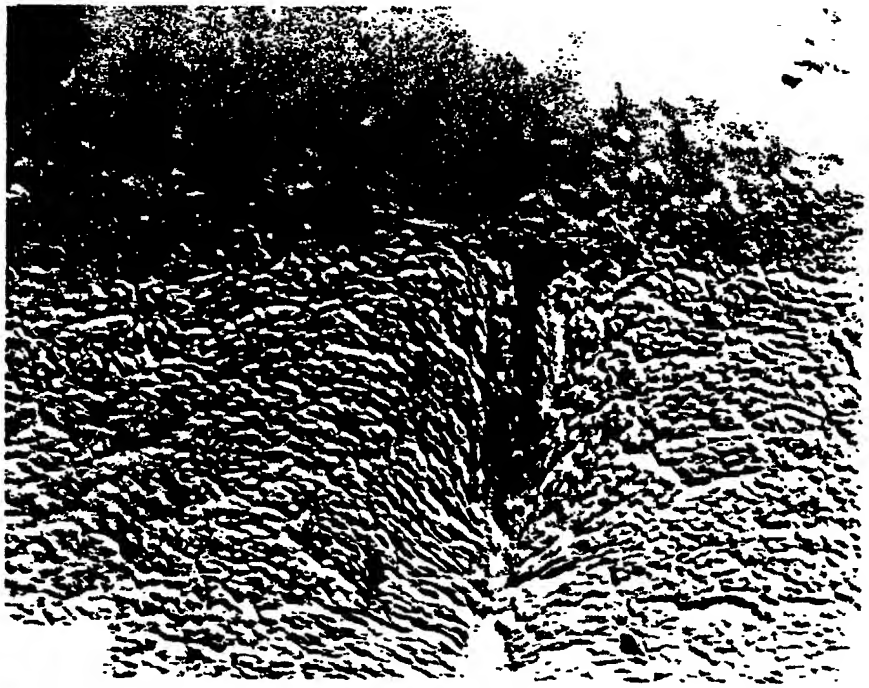


FIG. 11

FIG. 12A



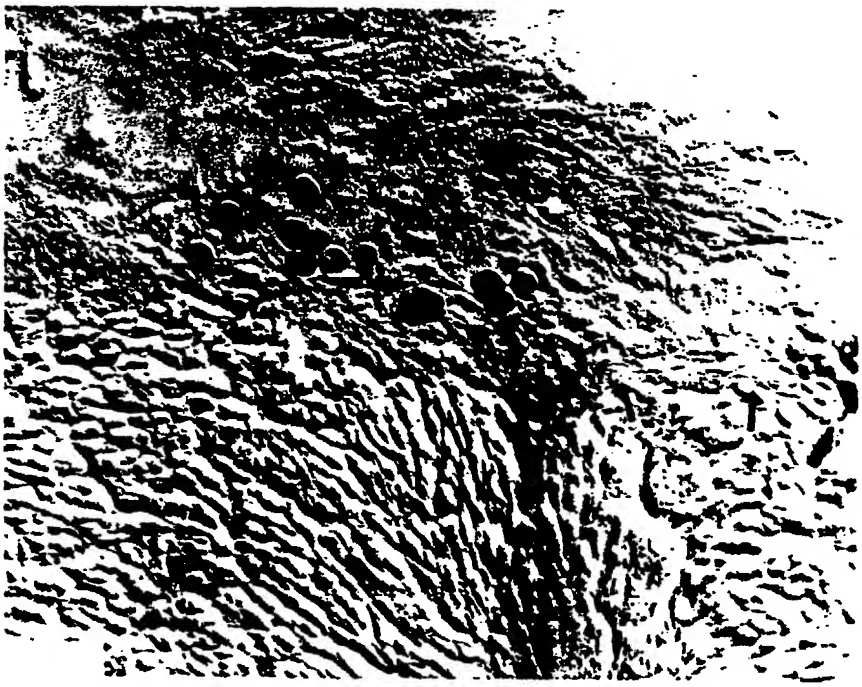


FIG. 12B

FIG. 13A

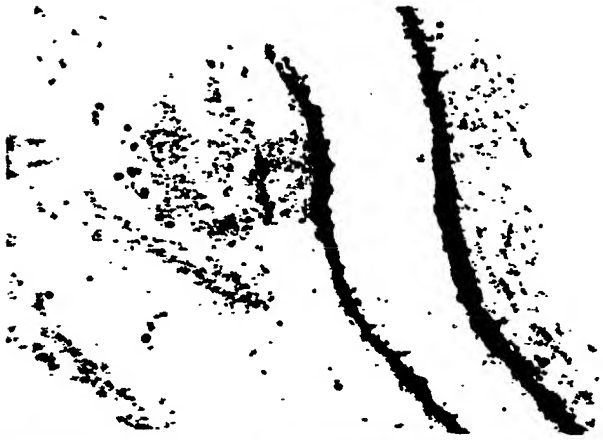


FIG. 13B

FIG. 13C

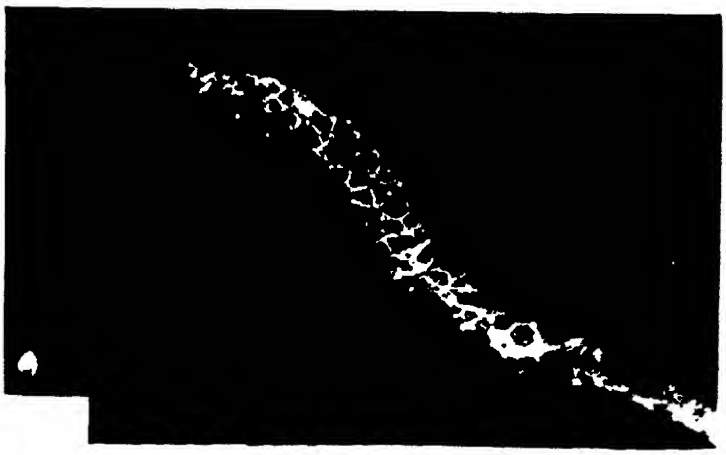


FIG. 14

FIG. 15A

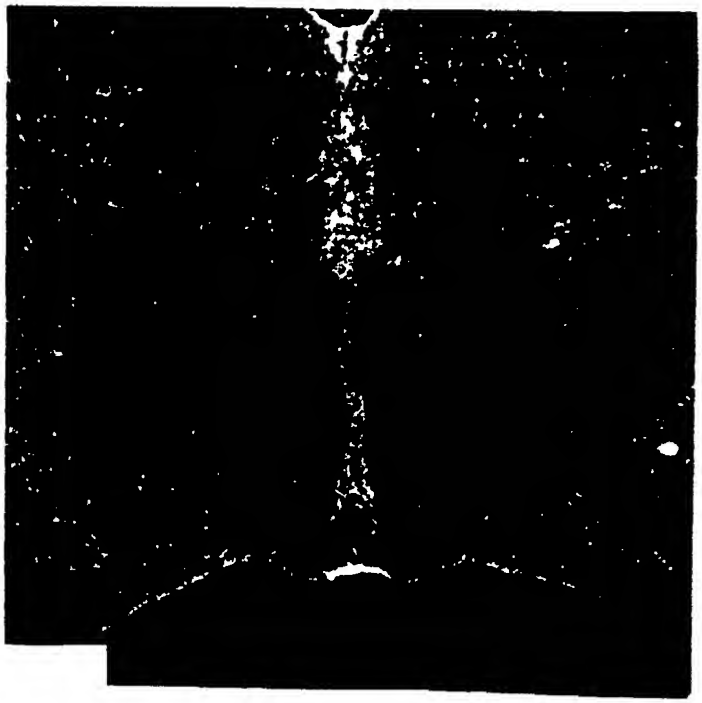


FIG. 15B

FIG. 16

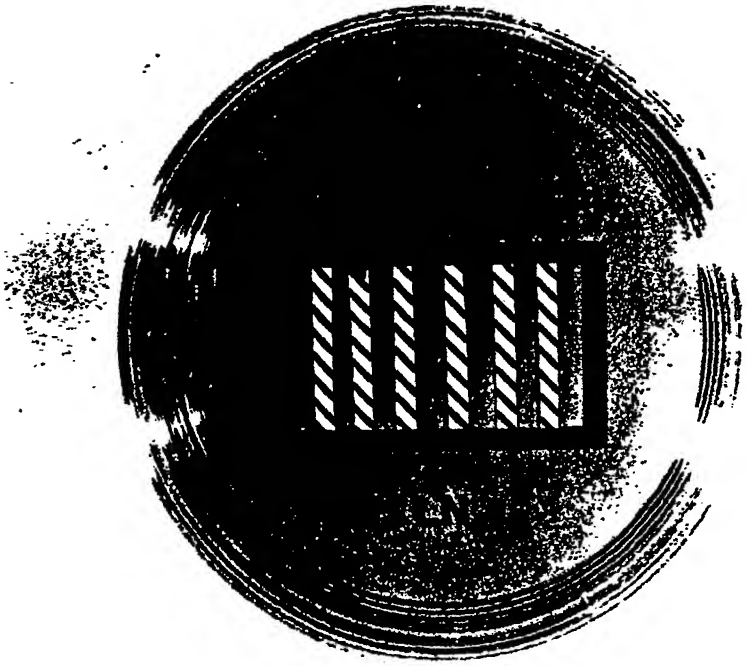


FIG. 17A

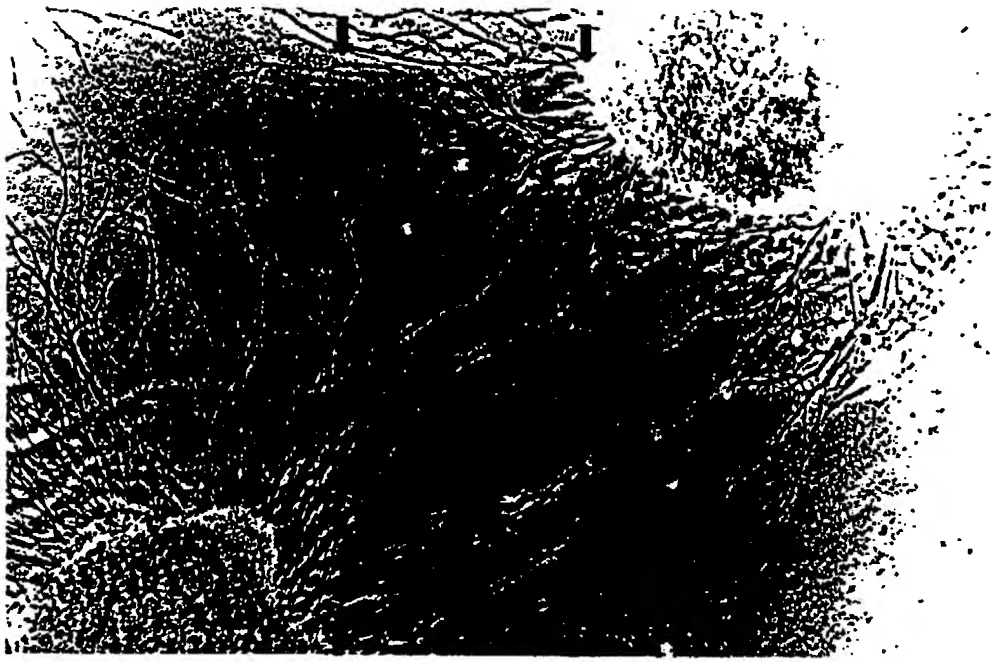


FIG. 17B

FIG. 17C

FIG. 18

FIG. 19

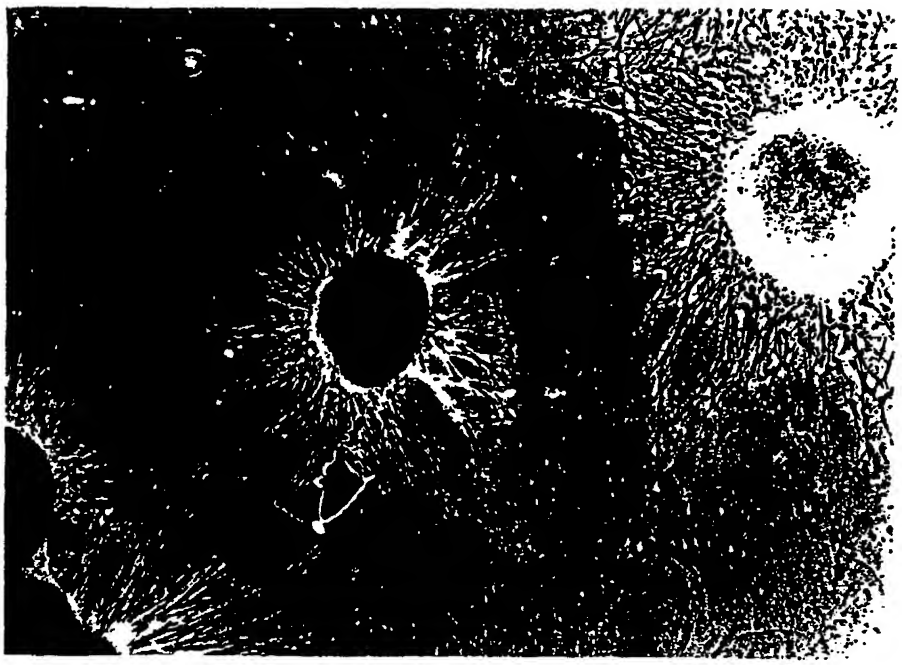


FIG. 20A

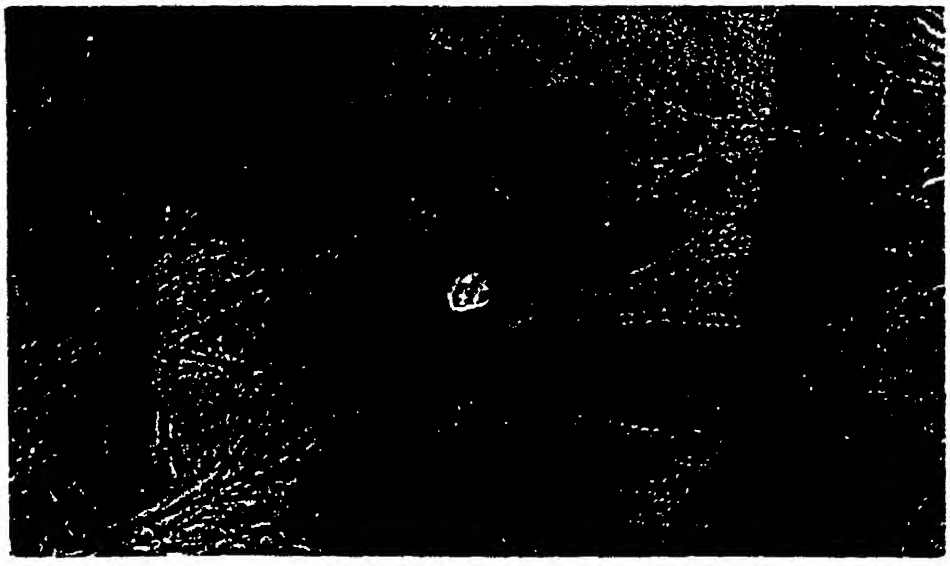


FIG. 20B

FIG. 21

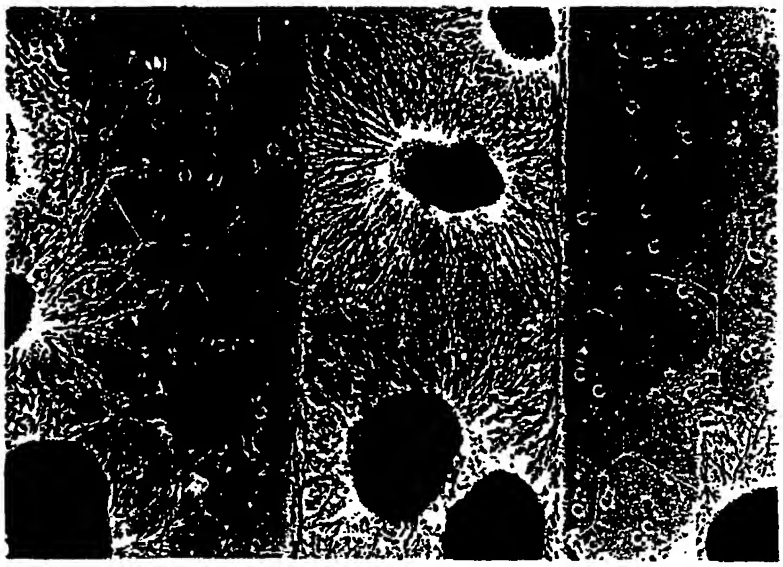


FIG. 22

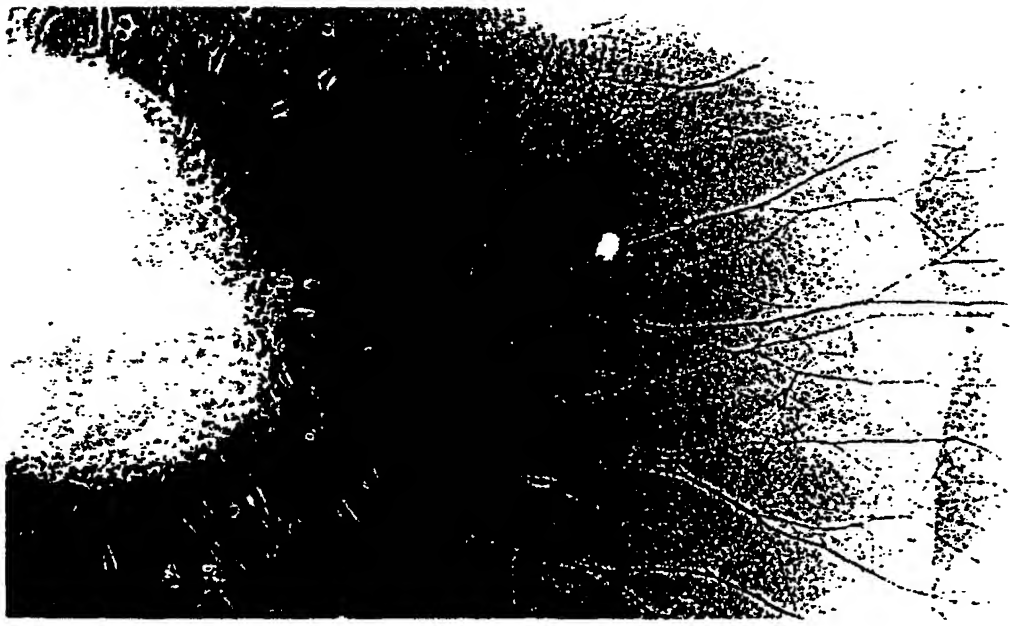


FIG. 23A

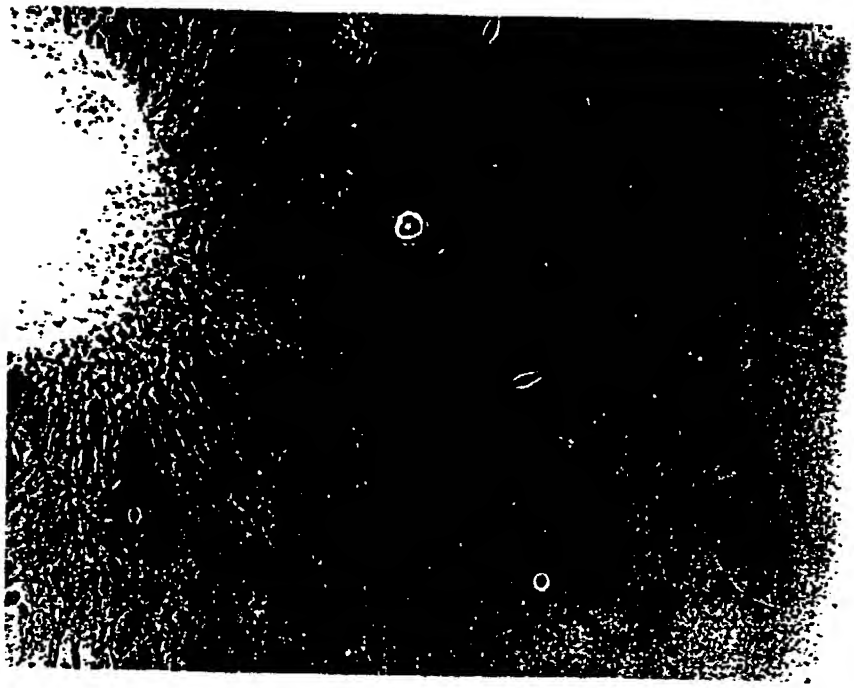
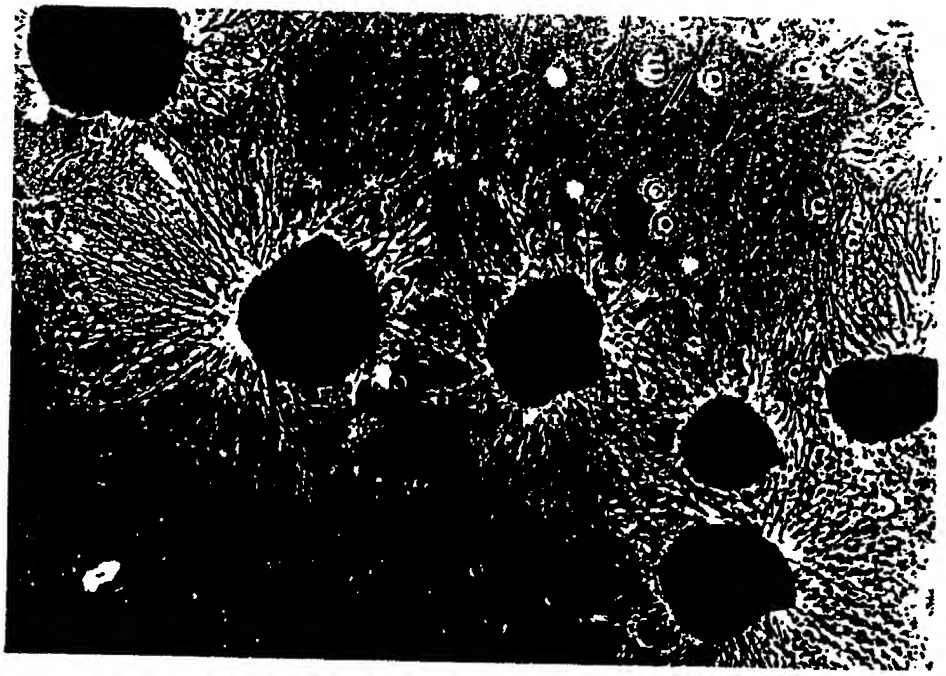


FIG. 23B

FIG. 24A



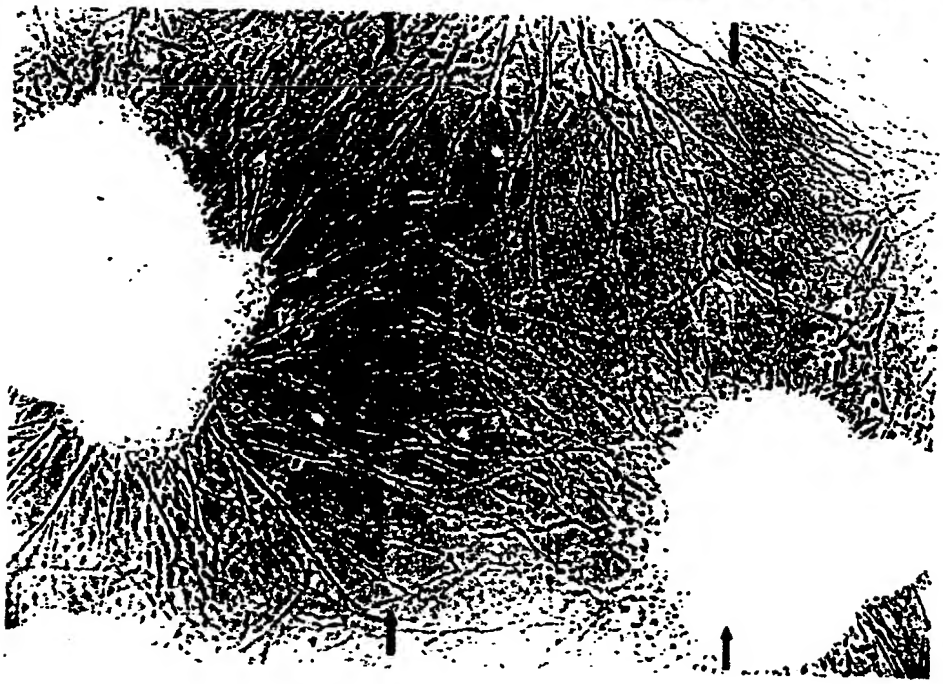
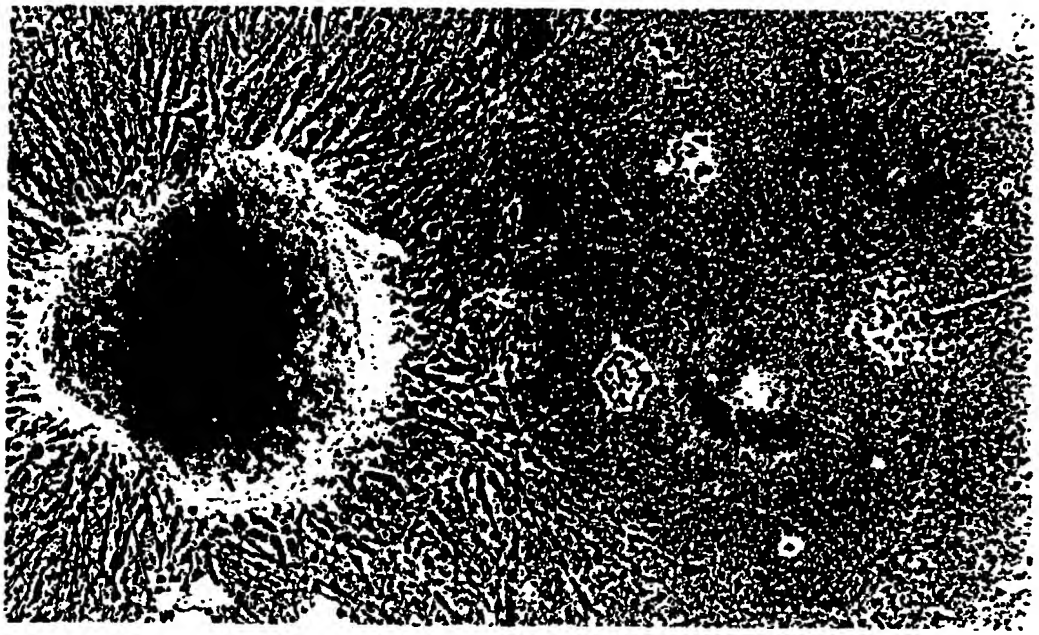


FIG. 24B

FIG. 25



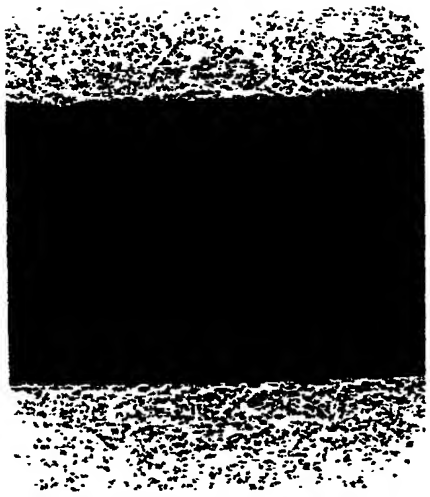


FIG. 26a

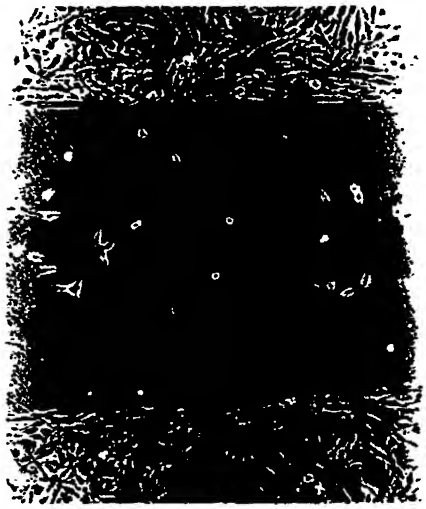


FIG. 26B

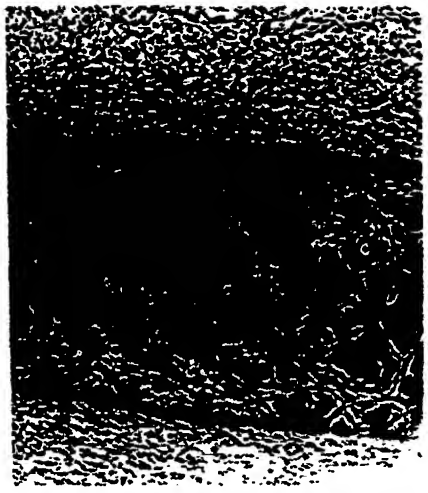


FIG. 26C

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06139

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5):A61K 31/715,31/725,37/48, 37/56. 39/395

U.S.CL.: 424/85.8 , 94.5, 94.61, 94.62, 94.63; 514/54, 56:

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S	424/85.8, 94.5, 94.61, 94.62, 94.63; 514.54, 56

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Automated Patent System and Chemical Abstracts Online Ters: "Keratan", "Chondroitin", "Dermatan", "Heparin", "Galactosidase", "Keratanase", "Chondroitinase", "chondroitin Lyase", "Hyaluronate".

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	US, A, 4,745,098 (Michaeli) 17 May 1988, see claim 5 and column 5, lines 36-42.	4-6,13, 128-133 43-49, 123-137, 139,140, 142,143
Y	US, A, 4,760,131 (Sundsmo et al.) 27 July 1988, see column 2, lines 37-46.	139,140, 142,143
X	US, A, 4,783,447 (Del Bono et al.) 18 November 1988, see column 7, lines 46-55.	7-10, 128-133
X	US, A, 4,710,493 (Landsberger) 01 December 1987, see columnm 3, lines 15-30.	4-6,13, 128-133
X Y	US, A, 4,640,912 (Hausman) 03 February 1987, see column 5, line 68- column 6, line 2 and claim 1.	4-6,13 43-49, 123-137

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 March 1991	25 MAR 1991

International Searching Authority	Signature of Authorized Officer
ISA/US	Mary E. Ceperley ebw

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	US, A, 4,083,960 (Yamashita et al.) 11 April 1978, see column 3, lines 61-64.	16
X	US, A, 4,696,816 (Brown) 29 September 1987, see column 2, lines 15-25.	30,31
X	US, A, 4,778,768 (Heinegard et al.) 18 October 1988, see column 8, lines 3-5.	30,31
X Y	US, A, 4,829,000 (Kleinman et al) 09 May 1989, see column 4, lines 15-18 and column 5, lines 37-46.	136,140 139,142 143
P,A	US, A, 4,956,348 (Gilbard et al.) 11 September 1990, see column 6, lines 7-25.	134,135
X Y	US, A, 4,808,570 (Michaeli) 28 February 1989, see claim 5.	4-6,13. 128-133 43-49,123 -127,139 140,142, 143
P,A	US, A, 4,945,086 (Benitz et al.) 31 July 1990, see column 1, lines 53-65 and column 8, lines 64-67.	27,30,31 128-133
X Y	US, A, 4,801,619 (Lindblad et al.) 31 January 1989, see the entire document.	134,135 141,144

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2 ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment.

1 ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice

2 ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3 ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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